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PARTICIPACIÓN DE LA INFLAMACIÓN EN LAS ALTERACIONES DEL SISTEMA NERVIOSO CENTRAL PRODUCIDAS POR LA INHALACIÓN DE O_3

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Me permito informar a usted que el Subcomité de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, en su sesión ordinaria del día 19 de enero de 2015, aprobó el jurado para la presentación de su examen para obtener el grado de **DOCTORA EN CIENCIAS** de la alumna **GONZÁLEZ GUEVARA EDITH** con número de cuenta **88229254** con la tesis titulada **"PARTICIPACIÓN DE LA INFLAMACIÓN EN LAS ALTERACIONES DEL SISTEMA NERVIOSO CENTRAL PRODUCIDAS POR LA INHALACIÓN DE O_3"**, realizada bajo la dirección del **DR. CARLOS HERLINDO PAZ TRES:**

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Sin otro particular, me es grato enviarle un cordial saludo.

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1. Resumen

La Organización Mundial de la Salud identificó a la contaminación del aire como el octavo factor de riesgo de mortalidad en los países de altos ingresos. La exposición a contaminantes ambientales como el ozono (O_3) aumenta el número de ingresos hospitalarios. El O_3 es un gas altamente reactivo que al tener contacto con las células epiteliales de las vías respiratorias, produce la formación de especies reactivas de oxígeno e inflamación. Más allá del sistema respiratorio, la exposición a O_3 también produce fatiga, letargo, dolor de cabeza y la disminución significativa del sueño de movimientos oculares rápidos relacionado con el incremento en el sueño de ondas lentas. Interesantemente, estos cambios en el sueño pueden revertirse por el tratamiento con indometacina, lo que sugiere que un mecanismo inflamatorio puede ser responsable de estos síntomas neurológicos. Para determinar si la respuesta inflamatoria puede ser el mecanismo mediante el cual el O_3 afecta los tejidos fuera del sistema pulmonar, se evaluaron factores inflamatorios tanto en los pulmones como en el cerebro. Se evaluó el efecto de la exposición a 1 parte por millón de O_3 en ratas durante 1, 3 o 6 h, así como en ratas expuestas 1 o 3 h al día durante cinco días consecutivos. Los resultados muestran incremento significativo en la concentración de TNF-α e IL-6 en los pulmones, así como aumento en la concentración de TNF-α, IL-6, aumento en el número de células inmunopositivas a NFκBp50 y aumento de la expresión de la proteína GFAP en la corteza cerebral. Estos resultados apoyan la hipótesis de que la respuesta neuroinflamatoria puede ser la responsable de los efectos reportados en el sistema nervioso central por la exposición a O_3.

2. Abstract

The World Health Organization identified urban outdoor air pollution as the eighth highest mortality risk factor in high-income countries. Exposure to ambient pollutants such as ozone (O_3) increases the number of hospital admissions. O_3 is a highly reactive gas that reacts with cells lining the airways, producing the formation of reactive oxygen species and inflammation. Beyond the respiratory system, O_3 exposure also produces fatigue, lethargy, headaches, and significant decrease in rapid-eye-movement sleep related to an increase in slow-wave sleep. Interestingly, these sleep changes can be significantly mitigated by treatment with indomethacin, which suggests that an inflammatory mechanism may be responsible for these neurological symptoms. To characterize the inflammatory mechanisms by which O_3 affects tissues outside the pulmonary system, we evaluated inflammatory factors in both lung and brain. Rats exposed to 1 part per million O_3 for 1, 3 or 6 h, as well as rats exposed daily for 1 or 3 h over five consecutive days, showed increases in TNF-α and IL-6 levels within the lungs as well as increases in TNF-α, IL-6, NF-κB p50 and GFAP levels in the cerebral cortex. These results support the hypothesis that the neuroinflammatory response may be responsible for the central nervous system effects of O_3 exposure.

3. Introducción

La contaminación atmosférica representa un grave de problema de salud, especialmente en los países en desarrollo, donde millones de personas están crónicamente expuestas a contaminantes del aire (Paz, 1997; Block y Calderón, 2009). El ozono (O_3), partículas materia (PM), y diversos materiales biológicos son los principales contaminantes del aire que respiramos y que causa daños graves en la salud. Se estima que la contaminación atmosférica representa el octavo factor de riesgo de mortalidad, lo que representa el 2,5% de todas las muertes en los países desarrollados (Narayan et al., 2010). Además, un aumento en la admisión de la gente en los hospitales, que se refleja por la pérdida productividad y de mano de obra, debido a la contaminación de O_3. (Jörres et al, 1996; Frank et al, 2001; Szyszkowicz et al, 2009). El O_3 puede ser generado de forma natural por la fotodisociación de moléculas de O_2 por los rayos ultravioletas del sol en longitudes de onda bajas. El O_3 También se pueden formar por descargas de alta tensión desde fricción del motor, señales de luz de neón, y otros equipos eléctricos como fotocopiadoras xerográficas, electrostática filtros de aire, las impresoras y los lugares de trabajo donde la soldadura es utilizado. Además, el O_3 se genera y se utiliza en la purificación de los sistemas de aire en edificios, en el control de crecimiento de hongos y bacterias en las plantas de almacenamiento en frío, en el tratamiento de aguas residuales, y en la purificación de agua potable.

4. Antecedentes

4.1 Contaminación ambiental

La contaminación atmosférica se define como la introducción de compuestos químicos, partículas de materia y partículas biológicas que modifican o alteran las características físicas y químicas de la atmósfera (EPA 2006). Los contaminantes atmosféricos se clasifican en primarios y secundarios, los primarios son aquellos que están presentes en la atmósfera tal y como fueron emitidos por la fuente, los secundarios son aquellos componentes primarios que sufren modificaciones químicas o que reaccionan entre sí. Los contaminantes ambientales son una mezcla de compuestos tales como: partículas de materia (PM), gases como ozono (O_3), monóxido de carbono (CO), óxido de azufre (SO), óxido de nitrógeno (NO), compuestos orgánicos (hidrocarburos aromáticos, endotoxinas) y metales (vanadio, níquel, manganeso) presentes en el aire (Akimoto, 2003). De estos compuestos, las PM y el O_3 son los implicados en problemas de salud más ampliamente asociados a enfermedades (Craig, 2008; Mills, 2009).

4.2 Ozono

El O_3 es el principal contaminante fotoquímico del aire (Oehme, 1996), se forma con facilidad en climas cálidos a partir de compuestos orgánicos volátiles (COV) y NO los cuales, al reaccionar en presencia de luz ultravioleta, contribuyen a la contaminación atmosférica de origen fotoquímico (Devlin, 1996). La mayoría de la gente alrededor del mundo está expuesta a altas concentraciones de contaminantes ambientales los cuales generalmente rebasan los estándares de seguridad establecidos (Akimoto, 2003). Los niveles de O_3 en la troposfera son el resultado de un ciclo químico que se presenta cuando la luz solar descompone el dióxido de nitrógeno (NO_2), en NO y oxígeno

atómico (O), y este último se une con el oxígeno del aire (O_2) para formar O_3 (Finlayson-Pitts, 1986; Derwent et al., 1996). Este ciclo "natural" se cierra cuando el O_3 reacciona con el NO y forma de nuevo NO_2 y O_2 (Fig.1). El estado de equilibro que tiene el ciclo del O_3, puede alterarse debido a la presencia de compuestos orgánicos volátiles (COV), ya que estos compiten con el O_3 por el NO disponible, de tal manera que parte del O_3 puede permanecer sin reaccionar y acumularse en la atmósfera. Se tiene conocimiento que la mayoría de los COV participan en la alteración del ciclo mencionado, con excepción de especies como el metano y los clorofluorocarbonos contribuyendo a la acumulación del O_3 (Canadian Council of Ministers of the Environment (CCME, 1996), European Climate Change Programme (ECCP, 2007). El O_3 es un gas incoloro de olor penetrante, altamente oxidante e inestable en altas concentraciones, es una molécula compuesta por tres átomos de oxígeno con alta energía de disociación (8547 cm^{-1}) (Banwell, 1983). Existe de manera natural en la tropósfera, sin embargo, se considera un contaminante cuando incrementa su concentración, representa un riesgo para la salud humana. Por lo que la última modificación a la norma oficial mexicana NOM-02-SSA1-1993, en salud ambiental determino que las poblaciones pueden estar expuestas a concentraciones por arriba de las 0.11 ppm durante periodos cortos de tiempo, pero que su promedio de ocho horas no debe exceder las 0.08 ppm; tal es el caso de las zonas metropolitanas de las ciudades de México y Guadalajara. El monitoreo del índice de calidad del aire durante el año 2011 reportó 146 días con categoría de calidad del aire MALA y 8 días MUY MALA por O_3; la concentración máxima de este contaminante se registró el día 12 de mayo de 2011 en la delegación Coyoacán con 184 ppb. De manera global, en 2011 solo 124 días registraron una calidad del aire favorable, en donde ninguno de los contaminantes registró una calidad del aire MALA o MUY MALA. Con respecto al cumplimiento de

las Normas Oficiales Mexicana de salud ambiental, en el periodo comprendido entre enero y diciembre se registró un total 449 horas con concentraciones de O_3 mayores a 110 ppb, mientras que el valor del quinto máximo para el promedio de 8 horas fue de 120 ppb.

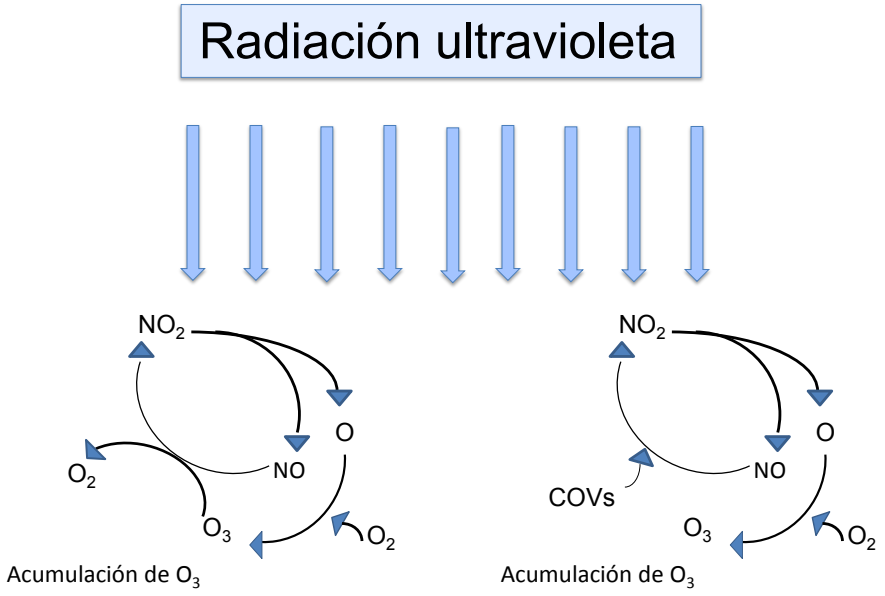


Figura 1. Ciclo fotoquímico de la formación de O_3 troposférico.

4.3 Efectos del O_3 en las vías respiratorias

El O_3 al igual que otros contaminantes atmosféricos penetra al organismo al inhalarlo (nariz, boca), así como por ojos y piel. La inhalación de O_3 genera efectos tóxicos sobre la salud (Menzel, 1993; Paz, 1997). El O_3 no es un radical libre, es una especie reactiva de oxígeno y un potente agente oxidante. Debido a que el O_3 es un gas altamente reactivo que no es capaz de penetrar al torrente sanguíneo, que reacciona directamente con los componentes de las células epiteliales de las vías respiratorias produciendo inflamación (Kafoury, 1999; Hollingsworth, 2007). Una vez inhalado, en el tejido pulmonar el O_3 interactúa con proteínas y lípidos modificándolos y generando compuestos que pueden ser tóxicos (Pryor, 1994; Pryor, 1995a). Como consecuencia de

la generación de estos productos oxidados, se activan macrófagos y se reclutan neutrófilos en el pulmón, además de presentar un incremento en la lipoperoxidación, inflamación de las vías respiratorias y disfunción de la inmunidad innata en el pulmón (Pryor, 1994; Pryor, 1995a; Hollingsworth, 2007). Estudios en el sistema respiratorio y cardiovascular demuestran que la inflamación y el estrés oxidativo son los principales mecanismos por los cuales la compleja mezcla de contaminantes del aire puede inducir daño (Riedl, 2008; Mills, 2009; Mühlfeld, 2008; Simkhovich, 2008).

La concentración inicial de O_3 y la dosis máxima en tejidos se localiza en la nariz y la barrera surfactante de las vías respiratorias. La tasa de absorción en la nariz de humanos es de alrededor de 40 a 65 % del O_3 inhalado durante la respiración (Gerrity, 1988; Kabel, 1994) sin embargo, solo una pequeña fracción (4-6 %) de la dosis total de O_3 reacciona con las membranas celulares (Pryor, 1992; Freeman, 1981), y comienza una cascada de reacción (Pryor, 1995a) que involucra la formación de especies reactivas de oxígeno (ERO)(Pryor, 2006), acumulación de derivados oxidados (Pryor, 1991) y procesos inflamatorios en las células epiteliales (Pryor, 1995b). Está descrita la transmigración de macrófagos, neutrófilos y otras células inmunes observada en lavados bronco-alveolares realizados en sujetos expuestos durante 4 h a 0.5 ppm de O_3 (Graham, 1988). También se ha observado atrofia de los cilios nasales e hiperplasia de las células basales en residentes de áreas contaminadas de la ciudad de México (Calderón-Garcidueñas, 1998). Pruebas de función respiratoria realizadas en sujetos sanos voluntarios que inhalaron una dosis de 0.25 ppm de O_3 muestran síntomas respiratorios severos que incluyen dificultad para respirar, irritación de las vías respiratorias, opresión en el pecho y tos (Calderón-Garcidueñas, 1998). También está descrito el aumento en el ingreso al departamento de emergencias en los hospitales debido a complicaciones respiratorias y exacerbación de asma (Szyszkovicz, 2009; Buchanan,

2008; WHO, 1987; WHO, 2006). Una sola exposición a O_3 causa cambios de la función respiratoria, incrementa la resistencia respiratoria, disminuye el volumen espiratorio forzado y la capacidad vital forzada ambos determinados durante un segundo (Aris, 1993; Rohr, 2002; McDonnell, 1997; Hatch, 1994). Además, se aumenta la reactividad de las vías aéreas, incrementa la permeabilidad a macromoléculas y la infiltración de neutrófilos en las zonas de contacto con el O_3 también se incrementa la secreción de moco en las vías respiratorias (Balmes, 1993; Koren, 1989). Los animales expuestos a O_3 muestran la presencia crónica de células inflamatorias y sus productos de secreción, estos procesos pueden contribuir a los cambios hiperplásicos y metaplásicos reportados en las vías aéreas de estos animales (Fanucchi, 1999). Los efectos sistémicos de la inhalación de O_3 están asociados con dolor de cabeza, alteraciones del sistema circulatorio y cambios en la función hepática (Last, 2005; Rahman, 1992) (Fig.2).

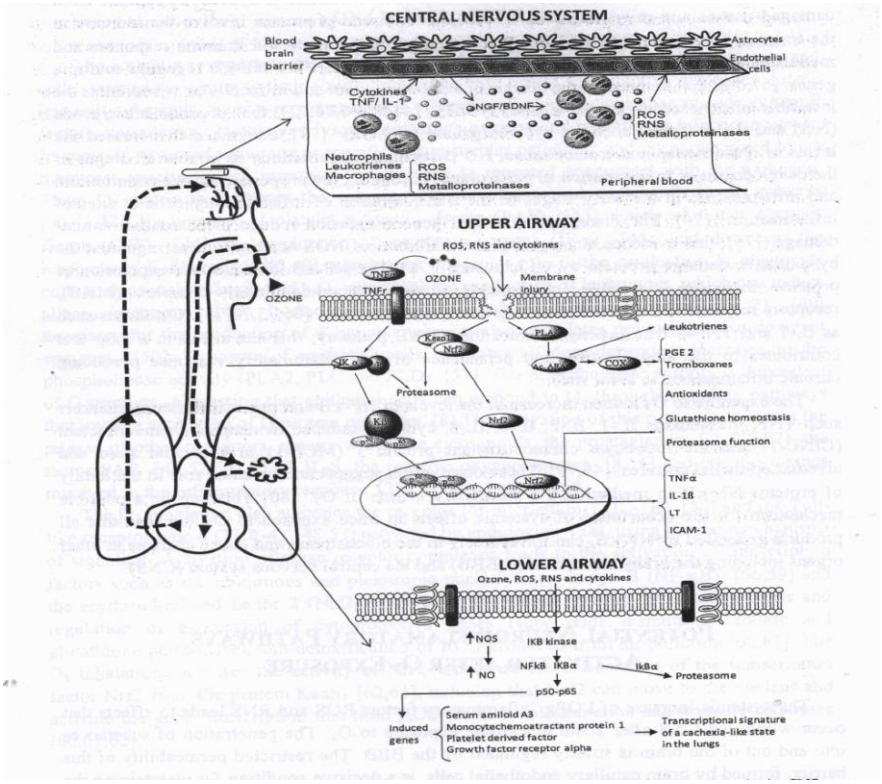


Figura 2. Vías de señalización implicadas en los daños producidos por la exposición a

O_3 y sus efectos moleculares en el SNC. (González, E., 2013)

4.4 Efectos del O_3 en el corazón.

Las enfermedades del corazón se desarrollan como resultado de complejas interacciones entre los genes y el ambiente. En los últimos 50 años, el entendimiento de las variadas causas y manifestaciones de enfermedades cardiacas se ha incrementado encontrando una fuerte asociación entre las exposiciones cortas a O_3 (uno o dos días) con los infartos a miocardio (Ruidavets, 2005; Bhatnagar, 2006). En particular, se consideran los efectos en el tono vascular, control de presión arterial, control autonómico de frecuencia cardiaca y concentración sérica de marcadores inflamatorios (Vaughan, 1984; Bhatnagar, 2004). El O_3 es un contaminante del aire altamente oxidante, estudios epidemiológicos muestran que los niveles de O_3 correlacionan con la morbilidad y mortalidad por causas respiratorias y cardiovasculares (Bell et al., 2004; Brook et al., 2010; Ito et al., 2005; Peters et al., 2001; Peters et al., 2004). Sin embargo un número de reportes sugiere un efecto independiente del O_3 en la mortalidad (de Almeida et al., 2011; Gryparis et al., 2004; Halonen et al., 2011; Ren et al., 2008; Stafoggia et al., 2010), el infarto agudo de miocardio (Halonen et al., 2011; Ruidavets et al., 2005), y el arresto cardiaco (Ensor et al., 2013). Sin embargo aún no es claro cómo la exposición a O_3 puede desarrollar eventos cardiovasculares fatales y no fatales.

4.5 Efectos del O_3 en hígado.

El hígado, es otro órgano afectado por la inhalación de O_3. Se observó en ratones expuestos a O_3 una regulación a la baja o decremento de genes para el citocromo P450 en el hígado la cual, parece ser debida a los productos de reacción del O_3 generados en el pulmón (Takahashi et al., 1985). La mayoría de los transcritos para P450 son regulados por esteroles e hidroxylasas (1A2, 2A4, 2D10, 2D11, 7B1, 8B1 y 17A1)

involucrados en la producción del ácido biliar y las vías metabólicas de hormonas esteroides (Last et al., 2005). Recientemente, con el uso de los análisis de expresión génica global, los investigadores encontraron que los hígados de ratones C57BL/6 expuestos en forma aguda a la inhalación de O_3 tienen una baja regulación de los genes relacionados con los lípidos, ácidos grasos, y metabolismo de los hidratos de carbono que se relacionan con caquexia (Last et al., 2005). La transcripción de varias moléculas de ARN mensajero (ARNm) que codifican para enzimas del metabolismo de xenobióticos se redujo en los hígados de los ratones expuestos a O_3. Varios genes hepáticos de interferones (IFN) se disminuyen con la exposición a O_3; los investigadores sugirieron que el IFN puede actuar como la molécula de señalización entre el pulmón y el hígado (Last, 2005; Takahashi, 1985). Además, ratones expuestos a O_3 prolongan el efecto del pentobarbital (Graham, 1981) y deterioro del metabolismo hepático de drogas (Ibrahim, 2010). No se conoce aún el mecanismo por el cual el O_3 induce estas alteraciones, sin embargo, se ha postulado que moléculas de señalización de larga vida, producida por células inflamatorias o epiteliales en el pulmón como respuesta al daño inducido por O_3 podrían ser las responsables de las alteraciones encontradas en el hígado. (Last et al., 2005).

4.6 Efectos del O_3 en el Sistema Nervioso Central

Los componentes de la contaminación ambiental pueden ejercer sus efectos tóxicos en el sistema nervioso central (SNC). En las grandes ciudades, la alta concentración de emisiones contaminantes, ha generado un interés particular sobre los efectos que pudiera ocasionar una exposición aguda o crónica al O_3, sobre el SNC. La inhalación de O_3 induce mareos y dolores de cabeza según lo reportado en trabajadores expuestos a este gas (Folinsbee, 2000; Hackney, 1975). La exposición a O_3 genera

estrés oxidativo en algunos núcleos implicados en procesos de cognición y actividad motora en el SNC, por lo cual se ha propuesto como un modelo no invasivo para el estudio de la participación del estrés oxidativo en los procesos neurodegenerativos (Rivas-Arancibia et. al., 2003). Sin embargo, el incremento especies reactivas de oxígeno (ROS) y especies reactivas de nitrógeno (RNS) activan los sistemas antioxidantes que contrarrestan la peroxidación lipídica y minimizan el daño celular; cuando el balance entre los oxidantes y los sistemas antioxidantes se rompe, las células se encuentran en estado de estrés oxidativo (Sies, 1991).

El O_3 produce diversas alteraciones en el SNC de los humanos, incluidas alteraciones bioquímicas, tales como incremento en las concentraciones de noradrenalina y dopamina (González-Piña y Paz, 1997; Cottet-Emard et al, 1997), además de degeneración celular (Rivas-Arancibia et al, 1998; Rivas-Manzano et al, 1999; Calderón-Garcidueñas et al, 2003). Estas alteraciones pueden producir fatiga, cefalea, disminución de la capacidad motora y alteraciones en el ciclo sueño-vigilia (Huitrón-Reséndiz et al, 1994). La inhalación de O_3 induce cambios en el ciclo de sueño vigilia, observados tanto en ratas como en gatos expuestos a 0.5 o 1 ppm de O_3 en ambos estudios se encontró el incremento del sueño de ondas lentas (SOL) y la disminución del sueño paradójico o (REM) (Paz and Bazán, 1992; Arito, 1992).

4.7 Inflamación

Está documentado en diferentes especies el desarrollo de un proceso inflamatorio pulmonar asociado con la exposición a bajas concentraciones de O_3, caracterizado por un aumento en el número de macrófagos, neutrófilos y eosinófilos (Bocci, 1998; Joad, 2000). Estas células liberan mediadores químicos como PGD2, LTs, tromboxano A2, factor de agregación plaquetaria y citocinas pro-inflamatorias como

TNF-α, IL-1, así como fibronectina, óxido nítrico, peróxido de hidrógeno y anión superóxido, todos ellos implicados en la patogénesis del daño tisular e hiperreactividad de las vías aéreas (Bocci, 1998; Joad, 1996; Grimes, 2011;Song, 2011; Gómez-Mejiba, 2009; Wright, 1994; Eling, 1988; Hunter, 1985).

La inflamación es la respuesta del sistema inmunológico de un organismo, al daño causado a sus células y tejidos vascularizados por patógenos bacterianos y por cualquier otro agresor de naturaleza biológica, química, física o mecánica. Aunque dolorosa, la inflamación es, normalmente, una respuesta reparadora; y un proceso que implica un gran gasto de energía metabólica. La inflamación es generada por citocinas y quimocinas pro-inflamatorias. Las citocinas son polipéptidos solubles de entre 8 y 60 KDa que regulan el crecimiento, la diferenciación y función de varios tipos celulares. La mayoría de las citocinas han sido asociadas con la regulación de procesos de inmunidad e inflamación, dentro del sistema inmune, sus efectos son ejercidos generalmente de manera parácrina o autócrina (Turnbull y River, 1999; Ambrosini and Aloisi, 2004). Se ha observado que el incremento de citocinas induce una cadena de reacción pleiotrópica y redundante estimulando a su vez, la síntesis de factores anti-inflamatorios. Los factores pro-inflamatorios como la interleucina 1 (IL-1), el factor de necrosis tumoral (TNF) y la interleucina 6 (IL-6) son los iniciadores de ésta cascada (Bartfai y Schultzberg 1993; Quinton et al, 2004; Chavarria y Alcocer-Varela, 2004). En la actualidad, están identificadas numerosas moléculas que poseen un papel muy importante en la inflamación. Dentro de éstas está el factor de necrosis tumoral (TNF), interleucina 1 (IL-1), interleucina 6 (IL-6), quimocinas, cicloxigenasa-2 (COX-2), 5-lipoxigenasa (LOX), metaloproteasas de matriz (MMP), factor de crecimiento endotelial vascular (VEGF), y moléculas de adhesión celular (Bharat y Prashasnika, 2009; Moalem et al., 1999; Martino, 2004).

Estas citocinas liberadas en el torrente sanguíneo pueden generar una respuesta inflamatoria sistémica; cuando estas citocinas llegan al sistema nervioso central, los astrocitos y algunos tipos neuronales son estimulados y secretan otras citocinas incrementando así, el proceso inflamatorio (Turnbull y River, 1999).

4.8 Inflamación en el Sistema Nervioso Central

Las citocinas secretadas por el sistema inmune de manera sistémica no pueden atravesar libremente la barrera hematoencefálica (BHE) sin embargo, existe evidencia que las moléculas circulantes producidas por exposición aguda o crónica a O_3 atraviesan la barrera por tres vías de entrada 1) sitios de la BHE dañada (Calderon-Garcidueñas et al., 2002); 2) los plexos coroideos (Calderon-Garcidueñas et al., 2008); 3) los órganos circunventriculares (Banks, 2005). Además del transporte activo que realizan los transportadores de las células endoteliales adyacentes a la BHE (Abbott y Friedman, 2012). Las células adyacentes a la BHE producen citocinas *de novo* lo que incrementa y mantiene la producción de estos factores inflamatorios, los cuales activan directa o indirectamente a neuronas, astrocitos y microglia (Turrin y Rivest, 2004). Las células gliales son sensibles a citocinas y marcadores inflamatorios así como a derivados de estrés oxidativo los cuales pueden incrementar en respuesta a diferentes estresores así como a la contaminación ambiental (Block y Calderón, 2009). Los astrocitos expresan receptores Tool Like (TLRs) los cuales, al activarse inducen la producción de mediadores inflamatorios incluyendo citocinas que amplifican la respuesta inmune local y modifican la permeabilidad de la BHE (Brambilla et al., 2005; Bsibsi et al., 2006). Cambios asociados con la pérdida de integridad de la BHE y la infiltración de leucocitos y eritrocitos en el parénquima cerebral se observan en sujetos expuestos a contaminantes atmosféricos, incluyendo el O_3 (Calderón-Gracidueñas et al., 2008). Por

consiguiente, un compromiso de la función en la BHE acompaña muchos trastornos neurológicos y está estrechamente relacionado con los procesos inflamatorios en el cerebro iniciados por infiltración de leucocitos de la sangre y/o la activación de las células gliales. Esos procesos inflamatorios contribuyen a la severidad y pronóstico de numerosos trastornos neurológicos y ambos pueden ser tanto la causa como el resultado de la disfunción en la BHE (de Vries et al., 2012).

Debido a que el O_3 inhalado se destruye al contacto con las células epiteliales de las vías respiratorias, no es directamente este quien produce los efectos extra-pulmonares. Se han postulado dos posibles mecanismos de daño inducido por O_3: 1) los radicales libres generados por la inhalación de O_3 como responsables de estos efectos sin embargo, la vida media de estos compuestos es muy corta (milisegundos en algunos casos) y además, después de la inhalación de O_3 se incrementa la cantidad de enzimas anti-oxidantes. 2) La respuesta inflamatoria en las vías respiratorias inducida por la inhalación de O_3 podría ser la responsable de estos efectos adversos (Martínez-Lazcano, 2013). Este podría ser el mecanismo por el cual la inhalación de O_3 induce daños extra pulmonares debido a que la administración de indometacina, un antiinflamatorio no esteroideo es capaz de revertir los cambios en el sueño de ratas expuestas a O_3 (Rubio y Paz, 2003). Considerando que el SNC es capaz de responder a estímulos inflamatorios periféricos, generando una respuesta neuroinflamatoria (Lacroix et al, 1998; Nadeu y Rivest, 2000). En este proceso inflamatorio, todos los componentes celulares del SNC pueden producir mediadores inflamatorios y expresar receptores a factores como las citocinas de manera constitutiva (García-Bueno, 2008; Plata-Salaman, 1991). La respuesta inflamatoria puede incrementar la concentración de algunos neurotransmisores como la noradrenalina que regula la activación del eje hipotálamo-pituitario-adrenal (HPA) (Morilak et al., 2005; Elenkov et al, 2000). Además, la

noradrenalina, incrementa la expresión de la proteína IκBα, la cual participa en un complejo proteico que recluta al factor NFκB, e impide que éste factor se traslade al núcleo y transcriba genes pro-inflamatorios (Feinstein et al, 1993; Galea y Feinstein, 1999; Gavrilyuk et al, 2002). El factor de transcripción NFκB participa en la regulación de la respuesta inflamatoria y la respuesta inmune, previene la apoptosis celular en respuesta a estrés celular (Wang et al., 2000a; Wang et al., 2000b; Deveraux y Reed, 1999). El NFκB incrementa la expresión de genes para proteínas que incluyen citocinas, quimiocinas, el complejo mayor de histocompatibilidad (MHC), receptores requeridos para la adhesión y migración de neutrófilos (Verma et al 1995; Baeuerte, y Baltimore 1996; Baldwin 1996; Ghosh et al., 1998; Pahl, 1999). Las citocinas como la interleucina 1α (IL-1α) y el factor de necrosis tumoral β (TNF-β) pueden activar directamente la vía del NFκB lo cual, puede resultar en un loop que contribuye a la amplificación de la respuesta inflamatoria y la persistencia de la inflamación crónica en sitios locales. El factor NFκB también puede estimular la expresión de enzimas como la sintasa del óxido nítrico inducible (iNOS) que genera óxido nítrico (NO) y estimula la ciclooxigenasa inducible (COX-2) (Pahl, 1999). El incremento de citocinas induce la activación de la microglia y los astrocitos en el sistema nervioso incrementando la producción tanto de la proteína glial fibrilar ácida como de citocinas proinflamatorias. La activación de la respuesta inflamatoria incrementa la producción de IL-6, la cual se sintetiza en las células de la pituitaria anterior, hipotálamo, células de la microglia y astrocitos en respuesta a daño. (Ray et al, 1989; Vankelecom et al, 1989). El incremento en la producción de la IL-6 se ha asociado con alteraciones de la homeostasis tales como trauma, sepsis o enfermedades inflamatorias. (Chai et al., 1996; Kozak et al., 1998; Oka et al., 2001; García-bueno 2008). La IL-6 en el SNC induce la producción de inmonuglobulinas (Fontana et al, 1989), estimula la secreción de la hormona liberadora

de corticotropina (Naitoh et al, 1988; Woloski et al, 1985), la secreción de la hormona de crecimiento, hormona luteinizante y prolactina de las células de la pituitaria anterior. También la IL-6 suprime la ingesta de comida durante la noche mientras que la incrementa durante el día, además de reducir la ansiedad (Butterweck et al., 2003). La IL-6 puede ser inducida por una variedad de moléculas entre ellas otras citocinas pro-inflamatorias como la IL-1 y el TNF-α (Lucas et al., 2006).

El TNF está implicado en la patogénesis de varias condiciones neurológicas, su producción se incrementa en el SNC después de un daño traumático, isquémico, infeccioso, neurodegenerativo o por enfermedades autoinmunes (Wang, 2002a; Beutler et al, 1987; Beutler et al, 1988). Las células de la microglia y los astrocitos sintetizan TNF-α y TNF-β de manera constitutiva. Se expresa en el hipotálamo, el tallo cerebral y otras regiones cerebrales (Lieberman et al, 1989; Righi et al, 1989; Robbins et al, 1987; Robbins et al, 1994). Ambas moléculas se unen al mismo receptor produciendo respuestas similares. El TNF es capaz de inducir fiebre a través de efectos directos en las neuronas hipotalámicas y de manera indirecta por la inducción de la liberación de la IL-1. La administración de IL-1β, TNF-α e IL-6 induce sueño de ondas lentas en conejos (Borbely y Tobler, 1989; Shoham et al, 1987).

La IL-1 es un importante mediador de neurodegeneración inducido en varios eventos de neuroinflamación (Rothwell y Luheshi, 2000). Diversos reportes señalan efectos neuromoduladores de la IL-1, incluyendo la estimulación del sistema central noradrenérgico (Kabiersch et al, 1988) y la estimulación el receptor rhIL-1β, la IL-1 puede regular la liberación y metabolismo de la noradrenalina en el hipotálamo (Kabierrsch et al, 1988; Palazzolo et al, 1989), induce la expresión de factores de crecimiento, disminuye la liberación de glutamato, modula la respuesta neuronal a NMDA y glicina, aumenta los efectos del GABA e incrementa la activación de la iNOS

(Lucas et al., 2006; Johnson et al., 2005; Dinarello, 2009) (Fig.3).

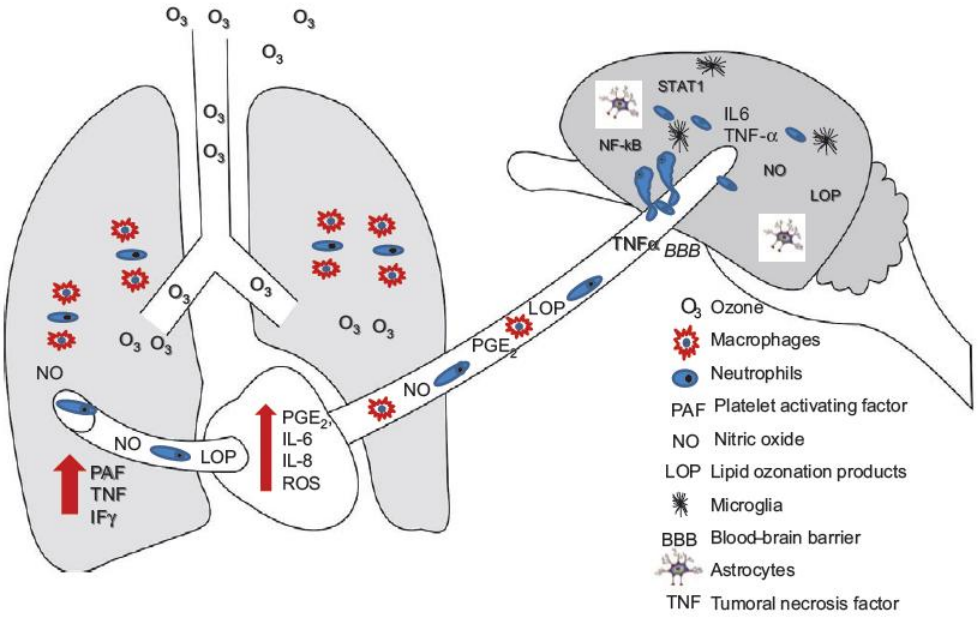


Figura 3. Activación de astrocitos y microglia inducida por la exposición a O_3 (Martínez-Lazcano et al., 2013).

La inyección sistémica o central de TNF o IL-1 aumenta la duración del sueño no MOR y las ondas delta registrado en electroencefalografía en: ratas, ratones, conejos, humanos, monos, gatos y ovejas (Krueger 2007, Krueger 2008). La inhibición tanto de la IL-1β como del TNF-α resulta en una pérdida de sueño (Krueger et al., 2001). Adicionalmente cuando los receptores para citocinas son inhibidos por bloqueadores, antagonistas, receptores solubles o mediante modelos genéticos, la duración del sueño se ve reducida (Fang et al., 1998; Krueger et al., 2003).

5. Justificación

La contaminación atmosférica se ha venido incrementando a nivel mundial; en México, la calidad del aire se ha visto afectada por la presencia cada vez mayor de múltiples contaminantes (partículas suspendidas, NO_2, O_3), lo que representa un problema que afecta la salud y la calidad de vida de sus habitantes. El mecanismo por el cual el O_3 genera alteraciones en el SNC aún no está determinado. Sin embargo, la inhalación de O_3 induce inflamación de las vías respiratorias. Aunado a este hecho el estudio realizado por Rubio y Paz en el 2003, muestra que los cambios inducidos en el sueño por la inhalación de O_3 son revertidos con indometacina, un anti-inflamatorio no estereoideo ha sugerido que la inflamación inducida por el O_3 en las vías respiratorias puede generar una respuesta inflamatoria sistémica la cual puede generar los cambios descritos en el SNC.

6. Planteamiento del problema:

El mecanismo por el cual el O_3 afecta al SNC aún no está descrito, sin embargo, debido a las características de los factores pro-inflamatorios es factible que al incrementarse como respuesta a un evento inflamatorio periférico, puedan ser difundidos por el sistema circulatorio y llegar tanto a los plexos coroideos como a la zona de los órganos circunventriculares y penetrar al parénquima cerebral para inducir una respuesta neuroinflamatoria. Por lo cual el propósito de esta investigación es determinar si la exposición a O_3 que genera una respuesta inflamatoria en las vías respiratorias es capaz de estimular el incremento en la producción de estas citocinas en el SNC dando como resultado una respuesta inflamatoria o neuroinflamación.

7. Hipótesis

Hipótesis alternativa

Si la exposición a O_3 genera inflamación en las vías respiratorias, entonces es factible que los factores inflamatorios (citocinas) lleguen al sistema circulatorio y puedan alcanzar el sistema nervioso central, donde incrementan su concentración y provocan una reacción inflamatoria (neuroinflamación).

Hipótesis nula

Si la exposición a O_3 no genera inflamación en las vías respiratorias, entonces no es factible que los factores inflamatorios (citocinas) lleguen al sistema circulatorio y puedan alcanzar el sistema nervioso central, entonces no incrementan su concentración y no provocan una reacción inflamatoria (neuroinflamación).

8. Objetivos

Objetivo general:

Demostrar que la exposición a O_3 produce neuroinflamación a través de la producción de citocinas y otros factores pro-inflamatorios en el sistema nervioso central.

Objetivos particulares:

Evaluar si la exposición a O_3 produce cambios en la concentración de las citocinas pro-inflamatorias TNF-α e IL-6 en el pulmón y la corteza cerebral de ratas.

Evaluar si la exposición a O_3 produce la activación del factor de transcripción NFkB determinado por el incremento de células inmunopositivas de NFkB p50 que se traslada al núcleo.

Evaluar si la exposición a O_3 incrementa la expresión de la proteína GFAP en el sistema nervioso central.

9. Desarrollo experimental

9.1 Exposición a O_3

Se expusieron ratas macho de la cepa Wistar, con peso de 250 a 300 gramos, durante 1, 3, 6 horas (grupos de exposición aguda) y después de 1 y 3 horas durante 5 días (grupos de exposición crónica) a 1 parte por millón (ppm) de O_3. Las ratas recibieron un flujo constante de aire libre de contaminantes durante el mismo tiempo (ratas control). Inmediatamente después de la exposición, las ratas se sacrificaron por decapitación para obtener los cerebros, de acuerdo a la técnica descrita por Glowisky e Iversen (1966) se obtuvo la corteza cerebral en su porción motora y una muestra del lóbulo derecho del pulmón. Las estructuras se mantuvieron en congelación a –70 °C hasta su procesamiento para desarrollar las diferentes técnicas.

Es importante destacar que gran parte de la evidencia sobre las alteraciones causadas por O_3 en el SNC se generan a partir de modelos animales expuestos a dosis que exceden los límites permisibles en los seres humanos. Hatch et al. (1994) demostraron que los roedores requieren dosis mucho más altas en comparación con las dosis de exposición reportados en los seres humanos en los que se observaron trastornos neurológicos debidos a la exposición O_3 (Hatch et al., 1994). Estas diferencias entre especies son porque, durante la exposición a O_3, las ratas eliminan una fracción más pequeña de la cantidad inhalada de O_3 (40 - 47%) (Wiester et al, 1987, 1988.) Que lo que liberan los seres humanos (75% con inter-variaciones individuales) (Wiester et al., 1996a). Por lo tanto, la toxicidad del O_3 observada para una concentración dada en roedores subestima fuertemente el efecto observado para la misma dosis en humanos.

9.2 ELISA

Los tejidos obtenidos se homogenizaron en un buffer de lisis de Tris-HCl (Sigma) pH 8.0 40 mM, que contenía 5 M de guanidina (Sigma), e inhibidores de proteasas (Sigma).

La concentración de proteínas se determinó con el kit para cuantificación de proteínas por el método de ácido bicinconilico BCA de Pierce.

La determinación de TNF-α e IL-6 se realizó de acuerdo a las especificaciones del Kit de cuantificación para TNF-α invitrogen (Cat. No. KRC3012) y para IL-6 invitrogen (Cat. No. CRC0063).

Durante la primera incubación se colocó la muestra homogenizada (50 µl para la IL-6 y 100µl para TNF-α) o una dilución estándar en cada pozo en placas sensibilizadas con el anticuerpo de captura. Durante la segunda incubación se agregó un anticuerpo de detección biotinilado específico para IL-6 o TNF-α de rata. Después de remover el exceso del anticuerpo secundario, se añadió estreptavidina-peroxidasa para el caso de la IL-6 y avidina-peroxidasa para el TNF-α, las cuales se unen al anticuerpo biotinilado. Posteriormente se lavó para remover la enzima libre, se agregó una solución sustrato (3,3', 5,5' tetrametilbenzidina-TMB parala IL-6 y ABTS para el TNF-α), la cual reacciona con la enzima (estreptavidinaperoxidasa o avidina-peroxidasa) para generar el cambio de coloración. La intensidad de color es proporcional a la concentración del marcador en el espécimen y la absorbancia se midió empleando el espectrofotómetro TECAN modelo Sunrise (a 450 nm para la IL- 6 y a 405 nm con una corrección a 620nm para el TNF-α). A partir de las absorbancias obtenidas y empleando una función de ajuste a partir de concentraciones conocidas se determinó la concentración a medir expresada en pg/ml. El límite inferior de detección para IL-6 fue $\leq$ 3 pg/ml y para TNF-

$\alpha \leq 6$ pg/ml. Posteriormente tras la corrección por concentración de proteína se determinaron los pg/mg de proteína, de cada una de las muestras.

9.3 Inmonuhistoquímica

Inmediatamente después de la exposición a O_3 las ratas se anestesiaron profundamente y fueron inyectadas transcardiacamente con heparina (1000 U) a través del ventrículo derecho, las ratas se prefundieron con PBS durante 30 s, inmediatamente después se inyectó paraformaldehído al 4% a 4°C. El cerebro fue removido del cráneo y mantenido en sacarosa/ PBS al 30% como crioprotector durante 24 h. Posteriormente los cerebros se incluyeron en parafina y fueron cortados sagitalmente (5um) usando un microtomo. Las secciones se montaron en laminillas con Poly-L-lisina y se colocaron en una estufa a 65°C durante toda la noche, posteriormente se realizó la desparafinización con xileno y la rehidratación a través de gradientes de etanol hasta llegar a agua destilada. Las secciones se lavaron en buffer de fosfatos y se incubaron durante 48 h a 4°C con el anticuerpo policlonal de cabra anti-NFkB p50 (1:100, sc-114, Santa Cruz). Después de tres lavados, se agregó el anticuerpo secundario conjugado con biotina-peroxidasa de rábano. La peroxidasa se determinó en color café (marrón) con su sustrato tetrahidrocloruro de diaminobenzidina (DAB). Las secciones se contra-tiñeron con hematoxilina previamente a cubrirlas. El número de células inmunopositivas se cuantificó en tres diferentes áreas de la corteza parietal de cada rata; estás secciones se fotografiaron y se analizaron con un software Image-Pro Plus adaptado a un microscopio Olympus IX81-F3 equipado con una cámara digital Q-Imaging. Las secciones fueron fotografiadas con un objetivo 40x en un campo visual de 520 μm^2. Los valores se expresan como el promedio ± el error stándard.

9.4 Western blot

Las muestras de tejido cerebral que se utilizaron para western blot se homogenizaron con inhibidores de proteasas para evitar la degradación en un buffer de lisis (Tris-HCl 40 mM pH 7.4, 1 % de NP-40), posteriormente se sonicaron durante 20 segundos. De este homogenizado se tomo una alícuota para la cuantificación de proteínas. A 50 μg de proteína se agregó la misma cantidad de buffer muestra 2X (Tris 4X HCl/SDS, pH 6.8, glicerol, SDS, 2-mercaptoetanol, azul de bromofenol) para realizar la electroforesis y la trasferencia a membranas de nitrocelulosa. Después de tener las proteínas en la membrana se bloqueo con leche al 5 % durante 2 horas y se incubo con el anticuerpo primario GFAP , durante toda la noche y después se realizaron tres lavados de 10 minutos cada uno con leche al 1% y tween al 0.1%. Inmediatamente después, se incubo el anticuerpo secundario anti-mouse conjugado con peroxidasa (1:1000) durante 1 hora y se realizaron tres lavados de 10 minutos con leche al 1% y tween al 0.1%. Al terminar los lavados se incuba con luminol para posteriormente realizar el revelado de la membrana en placas fotográficas.

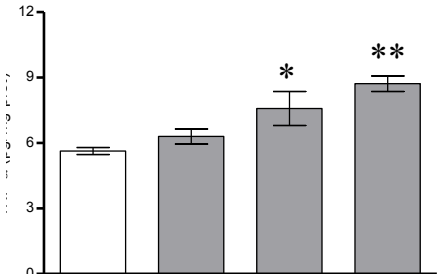
9.5 Análisis estadístico

Los resultados obtenidos de la prueba de ELISA, los datos de densidad relativa y el conteo de células inmunopositivas a NFκB se analizaron con una prueba de análisis de varianza y una prueba de significancia de Dunnet.

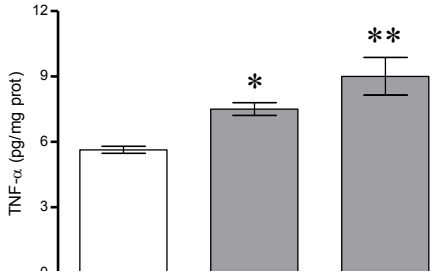
10. Resultados

La exposición a O_3 en incrementa la concentración de TNF-α en pulmón después de una hora sin embargo es significativa después de 3 y 6 horas de exposición ($F=8.699$, $df=3$, $p\leq0.05$), la exposición durante 1 o 3 horas durante 5 días consecutivos también muestra un incremento significativo en la concentración de TNF-α ($F=10.37$, $df=2$, $p\leq0.05$) después de la exposición a O_3 (Fig. 4A,4B)

A

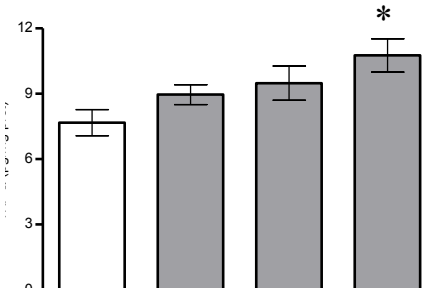


B

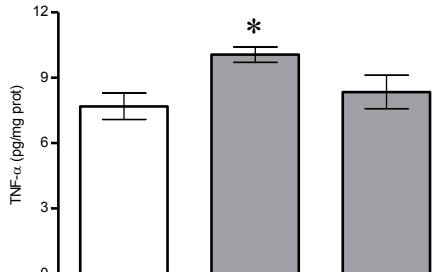


La determinación de TNF-α en la corteza cerebral de ratas expuestas a O_3 muestra su incremento después de 6 horas ($F=3.464$, $df=3$, $p\leq0.05$) y después de 1 hora ($F=4.184$, $df=2$, $p\leq0.05$) de exposición durante 5 días consecutivos (Fig. 5A, 5B).

A

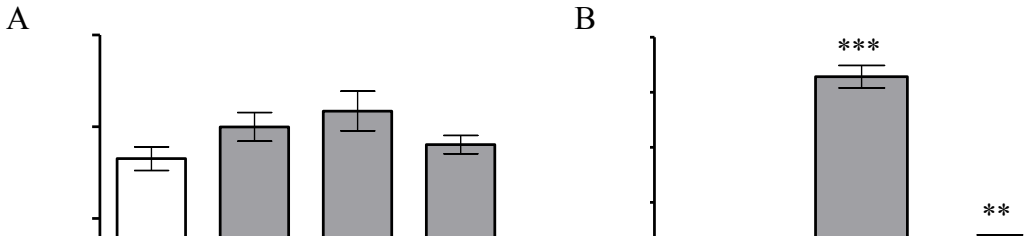


B

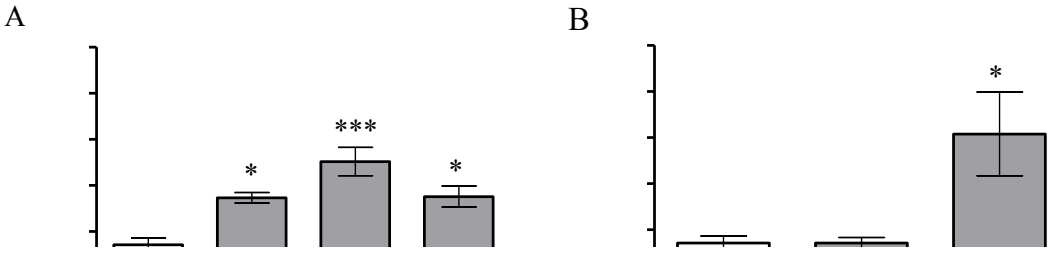


Después de la exposición aguda a O_3 en el pulmón, no encontramos cambios

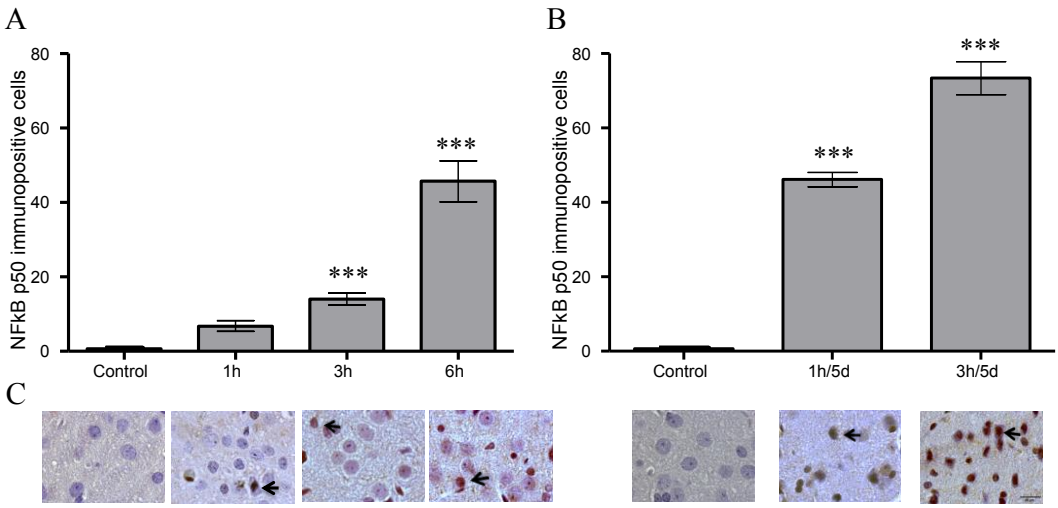
significativos sin embargo, en los grupos de exposición crónica encontramos cambios significativos después de 1 o 3 horas de exposición durante 5 días (F=248.1, d=f3, p≤0.05) (Fig. 6A, 6B).



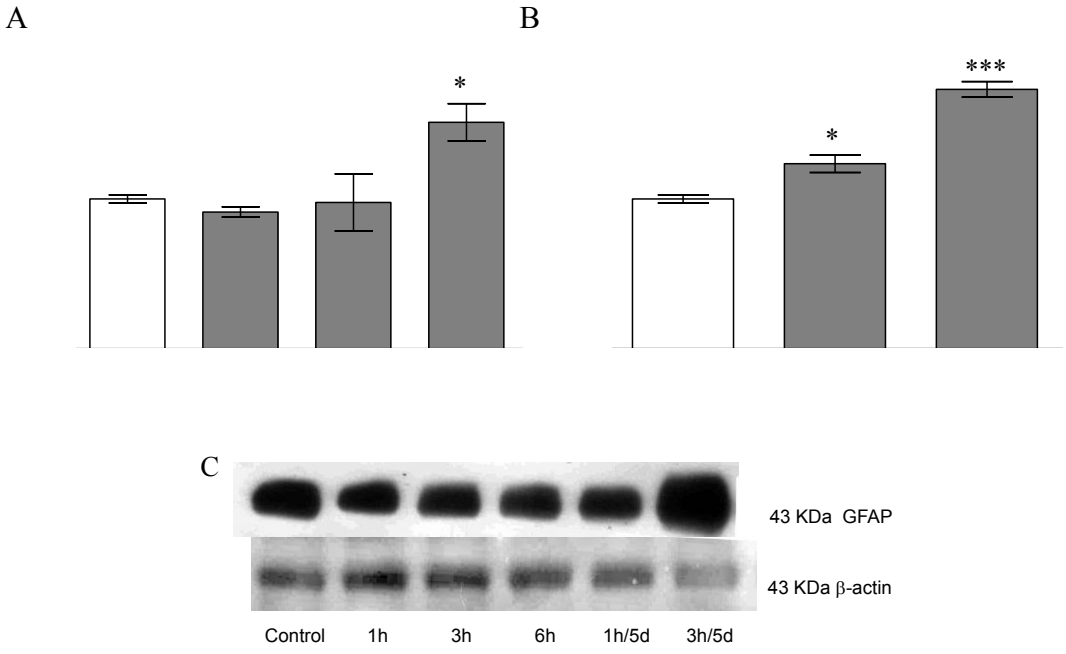
En la corteza cerebral encontramos cambios significativos en la expresión de IL-6 en los grupos de exposición aguda, después de 1, 3 y 6 h de exposición a O_3 (F=9.474, df=3, p≤0.05) y después de 3h de exposición durante 5 días (F=6.503, df=2, p≤0.05) (Fig. 7A, 7B).



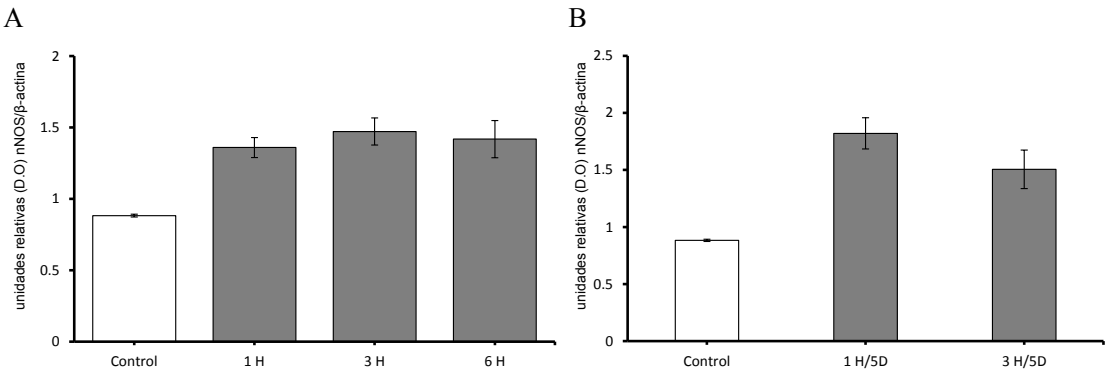
La exposición a O_3 induce el incremento significativo en la translocación al núcleo de NFκBp50 después de 3 o 6 horas de exposición (F=62.48, df=3, p≤0.0001) y después de 1 o 3 horas de exposición durante 5 días (F=163.9, df=2,p≤0.0001). (Fig. 8A,8B,8C).



La determinación de la proteína glial fibrila ácida (GFAP) presenta incrementos significativos después de 6 horas de exposición ($F=5.758$, $df=3$, $p\leq0.05$), y después de 1 o 3 horas de exposición durante 5 días ($F=54.76$, $df=2$, $p\leq0.05$) (Fig. 9A, 9B, 9C).



La expresión de la NOS neuronal incrementa después de 1, 3 o 6 horas y después de 1 o 3 horas durante 5 días de exposición a O_3. (Fig. 10A, 10B).



11. Discusión y conclusión

El O_3 troposférico es el que se encuentra próximo a la superficie. Es un gas tóxico debido a sus concentraciones elevadas, lo que genera repercusiones importantes en la salud humana. A medida que aumentan las concentraciones de ozono, los efectos (agudos y crónicos) en la salud de la población son cada vez mayores. Dichos efectos se pueden presentar en lugares en los que las concentraciones ya son elevadas debido a actividades humanas o que suben durante episodios de clima muy caluroso, los primeros síntomas son: tos, dolor de cabeza, náuseas, dolores pectorales, y sensación de asfixia.

Según la OMS, la mortalidad diaria y mortalidad por cardiopatías aumentan un 0,3% y un 0,4% respectivamente con un aumento de 10 μg/m3 en la concentración de O_3. Según estimaciones realizadas en el 2012, la contaminación atmosférica en las ciudades y zonas rurales de todo el mundo provoca cada año 3,7 millones de defunciones prematuras de las cuales el 80% de las defunciones prematuras relacionadas con la contaminación del aire exterior se deben a cardiopatía isquémica y accidente cerebrovascular, mientras que un 14% se deben a neumopatía obstructiva

crónica o infección aguda de las vías respiratorias inferiores, y un 6% a cáncer de pulmón (OMS, 2011; NOM-020-SSA1-2014).

Aunque la mayoría de los estudios sobre la exposición O_3 se han centrado en las vías respiratorias, algunas manifestaciones neurológicas, tales como letargo, fatiga y dolor de cabeza son reportados por habitantes de zonas contaminadas con altas concentraciones de O_3 (Dales, et al, 2009;. Winquist, et al, 2012.). Los estudios experimentales en diferentes cepas murinas muestran que la exposición a 0.60 a 1.00 ppm de O_3 disminuye la actividad motora (Tepper et al, 1982; Dorado-Martinez, et al, 2001; Rivas-Arancibia, et al, 2003), provoca deficiencias en la memoria y deterioro del comportamiento social (Avila-Costa, et al., 1999; Sorace, et al., 2001). La disminución significativa del sueño de movimientos oculares rápidos (MOR) y el aumento en el sueño de ondas lentas (SOL) se ha registrado en estudios electrográficos realizados en gatos y ratas después de la exposición a O_3 (Arito et al, 1992; Paz y Bazán-Perkins, 1992). Estos cambios en el sueño generados por la exposición a O_3 pueden ser debidos a cambios en la concentración de neurotransmisores en regiones cerebrales relacionadas con la regulación del sueño. El análisis neuroquímico demuestra que la exposición a 1.00 – 1.50 ppm de O_3 aumenta la concentración y el metabolismo de 5-HT, así como los niveles de dopamina y noradrenalina en el mesencéfalo de rata (Huitrón-Reséndiz, et al, 1994; Paz y Huitrón- Reséndiz, 1996; González-Piña y Paz, 1997) e inhibe la actividad de la tirosina hidroxilasa en el mesencéfalo después de la exposición 0.50 ppm O_3 durante 5 días (Cottet-Emard, 1997). La interrupción de los patrones de sueño debido a la exposición a O_3 puede ser revertida por el uso de un anti-inflamatorio la indometacina (Rubio y Paz, 2003). Debido a que el O_3 es un gas que no penetra más allá del tejido pulmonar, pero que reacciona en éste generando productos de reacción que

inducen una respuesta inflamatoria, generando una respuesta sistémica que puede extenderse hasta el SNC.

La respuesta inflamatoria consiste en una serie de reacciones inmunológicas que incluyen el aumento de la permeabilidad vascular y la liberación de derivados de lípidos, tales como eicosanoides, factor activador de plaquetas (PAF) o péptidos tales como IL-1, bradiquinina, y aminas como la histamina o 5-hidroxitriptamina a partir de tejidos dañados y células que migran (Aris et al., 1993; Ratto et al., 2006). La respuesta inflamatoria también implica el aumento de la transcripción del factor NF-κB que regula respuestas inflamatorias e inmunes y modula la apoptosis en respuesta al estrés celular (Wang et al., 2002b; Deveraux y Reed, 1999; Haddad et al., 1996). El NF-κB regula genes múltiples (Condorelli et al., 2002; Tian et al., 2002), que aumentan la síntesis de un potente oxidante y vasodilatador intracelular, la óxido nítrico sintasa inducible (NOS) (Grimes et al., 1983; Nakayama et al., 1992; Robbins et al., 1994; Kenyon et al., 2003; Fakhrazadeh et al., 2004), que producen óxido nítrico (NO) y la forma inducible de la ciclooxigenasa (COX-2) (Pahl, 1999). NO participa significativamente en casi todas las etapas del desarrollo de la inflamación, en particular en la regulación de las propiedades para el endotelio y la inflamación en las primeras etapas de la transmigración de células inflamatorias en sitios de inflamación (Rodríguez-Pascual et al., 1996). La interrupción de la expresión génica nNOS reduce el daño oxidativo naturales (Martínez-Lazcano et al., 2007), esta evidencia que sugiere que la inhibición de la actividad de la NOS se puede regular la producción del daño oxidativo por la exposición O_3 (Dorado-Martínez et al., 2001). El NF-κB aumenta la expresión de proteínas incluyendo citocinas, quimiocinas, receptores de complejos de histocompatibilidad principal (MHC), necesarios para la adhesión y migración de neutrófilos (Haddad et al., 1996; Pahl, 1999; Ghosh et al., 1998; Mills et al., 2009). Las

citoquinas tales como IL-1 y TNF-β pueden activar directamente la ruta de NF-κB; esto puede resultar en un círculo que contribuye a la amplificación y la persistencia de la respuesta inflamatoria produciendo inflamación crónica en sitios locales.

Las citocinas pro-inflamatorias pueden ser transportadas al SNC por la BHE permitiendo su penetración y acción (Teeling y Perry, 2009). Las citocinas regulan circuitos neurales implicados en varias respuestas y algunos procesos fisiológicos tales como la termorregulación, cambios en el comportamiento y la ingesta de alimentos (anorexia, pérdida de la actividad y la depresión) (Cartmell et al., 2000; Konsman et al., 2000) y los patrones de sueño. El consenso actual es que las citoquinas pro-inflamatorias, en particular, IL-1α, IL-1β, TNF-α e IL-6, generadas en la periferia, viajan al SNC, donde inducen la síntesis de más citoquinas y otras moléculas pro-inflamatorias (Bluthe et al., 2000a; Bluthe et al., 2000b; Ek et al., 2000). En las vías respiratorias, la exposición a O_3 induce aumento en los niveles de NF-κB y marcadores pro-inflamatorios como el TNF, interleucinas IL-1, IL-2, IL-6, IL-8, inducida por citoquinas quimiotácticas de neutrófilos (CINC-1) y la proteína quimiotáctica de monocitos 1 (MCP-1) en el líquido bronquial y cultivos de células de epitelio alveolar (Song et al., 2011; Manzer et al., 2008). Estas observaciones sugieren un papel importante en la familia de proteínas NF-κB en la mediación de los efectos pulmonares de O_3 (Li y Verma, 2002). Para caracterizar los posibles mecanismos inflamatorios a través de los cuales el O_3 afecta a los tejidos más allá del sistema pulmonar, se evaluó la respuesta inflamatoria en el pulmón y la corteza cerebral de ratas. En los pulmones, se encontró un aumento significativo de TNF-α después de la 3-h de exposición al O_3, y se observa esta respuesta después de 1 h o 3 h de exposición diaria durante 5 días. Además, un aumento de la concentración de IL-6 se encontró en el pulmón después de 1

h o 3 h de exposición O_3 durante 5 días. Estos resultados apoyan los informes anteriores de las vías respiratorias de los humanos y animales expuestos a O_3 (Devlin, et al, 1996; Cho, et al, 2007). En la corteza cerebral, encontramos un aumento significativo en el nivel de TNF-α después de una sola 6-h de exposición a O_3 y en ratas expuestas a este gas durante 1 h al día durante 5 días consecutivos. Además, un aumento significativo de la IL-6 fue encontrado después de la exposición O_3 durante 1 h, 3 h, 6 h o o durante 3 h por día durante 5 días consecutivos. Se sabe que tanto el TNF-α e IL-6 pueden cruzar la barrera sangre-cerebro para alcanzar el CNS por difusión simple o por medio de transportadores específicos localizados en las células endoteliales de la barrera sangre-cerebro (Banks, 2005). En el SNC, las citoquinas son capaces de estimular la liberación de IκBα de NFkB, el cual se transloca en las subunidades NFkBp50 y NFkB p65 al núcleo para activar la expresión de múltiples genes implicados en la respuesta inflamatoria (Stein, et al., 1993; Pahl, 1999; Cho, et al, 2007). Hemos encontrado aumentos significativos en NFkB p50 después de exposiciones individuales O_3 de 1 h, 3 h y 6 h, así como después de 1 h y 3 h exposiciones durante 5 días consecutivos. NFkB p50 también es capaz de inducir la expresión de GFAP (Sticozzi, 2013). GFAP entonces induce la expresión de varios genes implicados en la respuesta inflamatoria (Okada et al., 2006). Nuestros resultados mostraron que la expresión de GFAP aumentó después de 6 h de exposición o después de 1 h o 3 h de exposición durante 5 días consecutivos. Además, se ha demostrado que tanto TNF-α e IL-6 administrado intracerebroventricularmente pueden inducir incrementos significativos en SWS y disminuye en REM (Krueger, 2008; Dantzer y Kelley, 2007).

El incremento de citocinas induce inflamación tanto a nivel sistémico como en el SNC, nuestros resultados muestran que el incremento o activación de moléculas pro-inflamatorias, como TNF-α, IL-6, en el pulmón inducen el incremento de estás en el

SNC, además de factores de transcripción (NFκB) y activación glial (GFAP), como parte de la respuesta inflamatoria inducida por O_3.

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13. Anexos

13.1 Publicaciones derivadas de este trabajo.

13.1.1 Inhalation Toxicology 2014

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RESEARCH ARTICLE

Exposure to ozone induces a systemic inflammatory response: possible source of the neurological alterations induced by this gas

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Abstract

The World Health Organization identified urban outdoor air pollution as the eighth highest mortality risk factor in high-income countries. Exposure to ambient pollutants such as ozone (O_3) increases the number of hospital admissions. O_3 is a highly reactive gas that reacts with cells lining the airways, producing the formation of reactive oxygen species and inflammation. Beyond the respiratory system, O_3 exposure also produces fatigue, lethargy, headaches, and significant decrease in rapid-eye-movement sleep related to an increase in slow-wave sleep. Interestingly, these sleep changes can be significantly mitigated by treatment with indomethacin, which suggests that an inflammatory mechanism may be responsible for these neurological symptoms. To characterize the inflammatory mechanisms by which O_3 affects tissues outside the pulmonary system, we evaluated inflammatory factors in both lung and brain. Rats exposed to 1 part per million O_3 for 1, 3 or 6 h, as well as rats exposed daily for 1 or 3 h over five consecutive days, showed increases in TNF-α and IL-6 levels within the lungs as well as increases in TNF-α, IL-6, NF-κB p50 and GFAP levels in the cerebral cortex. These results support the hypothesis that the neuroinflammatory response may be responsible for the central nervous system effects of O_3 exposure.

Keywords

Brain, cytokines, GFAP, inflammation, NF-κB, ozone

History

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Introduction

The World Health Organization (WHO) identified urban outdoor air pollution as the eighth highest mortality risk factor in high-income countries (Narayan et al., 2010). Air pollution is composed of a diverse mixture of particulate matter, gases, organic compounds and metals. Of these compounds, particulate matter and ground-level O_3 are the most widespread health threats and have been heavily implicated in disease (Craig et al., 2008; Mills et al., 2009). Therefore, the WHO has established the O_3 exposure limit of 100 μg/m^3 [510 parts per million (ppm)] during 8 h daily for urban areas. Excessive O_3 in the air can have a marked effect on human health such as breathing problems, trigger asthma, reduce lung function and cause lung diseases (World Health Organization, 2005). Studies have demonstrated that O_3 exposure can also produce neurological symptoms, such as fatigue, lethargy and headaches (Dales et al., 2009; Winquist et al., 2012).

In animal models, the O_3 is usually administered in higher doses, i.e. the rats need be exposed to 2.0 ppm O_3 in order to achieve immunological changes in the bronchoalveolar lavage fluid (BALF) as those found in human exposed to 0.4 ppm (Hatch et al., 1994). Such difference has been attributed to high metabolism and increased concentration of endogen antioxidants in the rat (Kari et al., 1997; Slade et al., 1993). Though, minor O_3 concentrations have been used to produce physiological and biochemical changes in the rat brain (Arito et al., 1992; Huitrón-Reséndiz et al., 1994; Paz & Bazán-Perkins, 1992; Paz & Huitrón-Reséndiz, 1996). Also, significant teratogenic effects have been found in pups from mothers exposed to O_3 during pregnancy (Custodio et al., 2010; Haro & Paz, 1993; Kavlock et al., 1979, 1980; Rivas-Manzano & Paz, 1999).

It is known that O_3 react mainly with the lining cells in airways, inducing the secretion of cytokines and inflammatory factors (Graham et al., 1981; Ibrahim et al., 2010; Pryor et al., 1995a, 1995b; Takahashi et al., 1996). Changes in the sleep patterns of rats induced by O_3 exposure can be significantly inhibited by indomethacin treatment (Rubio & Paz, 2003), suggesting a neuroinflammatory response against O_3 exposure (Martínez-Lazcano et al., 2013). To characterize the inflammatory mechanisms through which O_3 affects tissues outside of the pulmonary system, we evaluated inflammatory factors in both lung and brain. Specifically, we decided to study the inflammatory mediators TNF-α, IL-6, NF-κB p50 and GFAP to determine the possible participation of the inflammatory response in CNS of rats exposed to 1 ppm O_3 during 1, 3 or 6 h and during 1 or 3 h for five consecutive days.

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Materials and methods

Animal care and treatment

We used male Wistar rats weighing 250–300 g. All efforts were made to minimize both the number of animals used and any potential pain or distress. The handling of all rats conformed to institutional guidelines that comply with national regulations (NOM-062-ZOO-1999) and international guiding principles (Council for International organizations of Medical Sciences, CIOMS). Rats were individually housed and allowed to move freely in transparent cages with corn-cob bedding at 23 ± 1 °C under a 12-h light–dark cycle (lights on at 07:00 h). The rats were allowed *ad libitum* access to food and water. The rats were then transferred individually to hermetic chambers (30 × 25 × 30 cm) supplied with unpolluted air (1.7 l/min) at the same climatic condition and free access to food and water. Subsequently, the chambers were provided with 1 ppm O_3 using a P15 TRIOZON generator (TRIOZON, Tlalnepantla, MX). The concentration of O_3 was measured and monitored constant using a Serinus 10 (Ecotech, Melbourne, AU) ultraviolet light analyzer. The rats were randomly divided into six independent groups ($n = 9$ in each): (i) control; (ii) 1 h O_3 exposure (1 h), (iii) 3 h O_3 exposure (3 h); (iv) 6 h O_3 exposure (6 h); (v) exposure to O_3 for 1 h daily for 5 consecutive days (1 h/5 d); and (vi) exposure to O_3 for 3 h daily for 5 consecutive days (3 h/5 d). The rats exposed to O_3 by 1 or 3 h during 5 consecutive days were exposed at the same time of day (09:00 h). Animals were sacrificed immediately following O_3 exposure, and the brains were extracted and dissected at low temperature to collect the cerebral cortex. At the same time, lung tissue was collected from the right middle lung lobe. Tissues were stored at −70 °C until analysis. For the immunohistochemical procedures, three rats per group were transcardially perfused. The brains were extracted and stored at −4 °C until histological processing.

Enzyme-linked immunosorbent assay

Tissues from the lung and cerebral cortex from three rats were homogenized in ice-cold lysis buffer containing 5 M guanidine HCl. After grinding, the samples were mixed at room temperature for 3 h; at this stage, the samples are stable and can undergo multiple freeze-thaw cycles. After obtaining the samples, we used commercially available enzyme-linked immunosorbent assay (ELISA) kits (KRC3011 and CRC0063, Invitrogen Corporation, Carlsbad, CA) to determine the concentration of TNF-α and IL-6 in the samples; cytokine levels were measured by monitoring optical density, according to the manufacturer's recommendations.

Western blotting analyses

Tissues from the lung and cerebral cortex from three rats were homogenized in ice-cold lysis buffer containing 40 mM Tris–HCl pH 8.0 (Sigma, St. Louis, MO), 1% IGEPAL (NP-40) detergent (Aldrich, St. Louis, MO), 10 mM sodium fluoride (Sigma), 1 mM sodium vanadate (Sigma) and protease inhibitors (Sigma). Following homogenization, the samples were sonicated for 20 s. Protein concentrations were determined using the bicinchoninic acid Protein Assay Kit (BCA, Pierce, Rockford, IL). For each sample, 50 µg of protein was separated on a 12% sodium dodecyl sulfate–polyacrylamide (SDS) minigel (Bio-Rad Laboratories, Inc., Hercules, CA) and transferred onto a nitrocellulose membrane (Bio-Rad). Next, the membranes were blocked with 5% fat-free dried milk and 0.1% Tween-20 in phosphate-buffered saline (PBS) solution for 1.5 h. The blots were then incubated overnight at 4 °C with a monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:200; sc-51601, Santa Cruz Biotechnology, Santa Cruz, CA). After the antibody incubation, the blots were washed three times (10 min each) with 1% fat-free dried milk and 0.1% Tween-20 in PBS. The blots were then incubated for 1 h with a peroxidase-conjugated secondary antibody (1:1000; Santa Cruz) and washed three times (10 min each) with 1% fat-free dried milk and 0.1% Tween-20 in PBS. The blots were developed using the Visualizer Western Blot Detection Kit (Millipore, Billerica, MA). The same blots were also incubated with an anti-actin antibody (1:1000; Chemicon, MAB1501; Millipore, Billerica, MA) as a loading control. Images from blots were digitally acquired with a Hewlett Packard M1120 scanner (Hewlett Packard Company, Palo Alto, CA), and densitometry analysis was performed in Sigma-Gel software (Cyber Innovations Corporation, Silicon Valley, CA). Densitometry results of the GFAP were normalized with respect to their β-actin control and data expressed as normalized optical density arbitrary units.

Immunohistochemistry

Rats from each group were anesthetized after O_3 exposure and transcardially injected with heparin (5000 U/ml) in PBS through the right ventricle for 30 s, followed by the fixative 4% paraformaldehyde (Sigma) at 4 °C. The brains were removed from the skull and post-fixed for 24 h in the same fixative at 4 °C. Brains were subsequently transferred into a 20% sucrose cryoprotective solution in PBS at 4 °C for 24 h. After these procedures, the brains were embedded in paraffin, and serial sagittal slices of 5 µm were made. The sections were baked overnight at 65 °C, followed by deparaffinization with xylene and rehydration through graded ethanol to distilled water. The sections were washed in PBS and incubated for 48 h at 4 °C with anti-NF-κB p50 goat polyclonal antibody (1:100, SC-114, Santa Cruz). After three washes, the appropriate biotinylated secondary antibody and avidin–biotin–peroxidase were added sequentially. The peroxidase was detected in brown with a diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Laboratories, Inc., Burlingame, CA). The sections were then counterstained with hematoxylin prior to coverslipping. The number of immunopositive cells was quantified in three different areas of the parietal cortex for each rat; these sections were photographed and analyzed with Image-Pro Plus software (Media Cybernetics, Rockville, MD) adapted to an Olympus IX81-F3 (Olympus Corporation, Tokyo, Japan) microscope equipped with a Q-Imaging digital camera kit. The sections were imaged with an objective 40× in a field of 520 µm^2. Values are expressed as the mean ± standard error.

Statistical analyses

Data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was carried out with one-way

analysis of variances, followed by Dunnett's *post hoc* test. For all types of experiments, $p \leq 0.05$ were considered significant. GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA) was employed for all analyses.

Results

To demonstrate the propagation of a systemic inflammatory response produced by O_3 exposure, the levels of TNF-α, IL-6, NF-κB p50 and GFAP in the lung and the cerebral cortex were evaluated. We found significant increases in TNF-α levels in the lung after 3 or 6 h of O_3 exposure ($F = 8.699$, df = 3, $p < 0.05$) and after 1 or 3 h of O_3 exposure for 5 consecutive days ($F = 10.37$, df = 2, $p < 0.05$) (Figure 1A). Significant increases in TNF-α levels were also found in the cerebral cortex after 6 h of O_3 exposure ($F = 3.464$, df = 3, $p < 0.05$) and after 1 h of O_3 exposure for 5 consecutive days ($F = 4.184$, df = 2, $p < 0.05$) (Figure 1B).

We did not find significant changes in IL-6 levels in the lungs of rats following single O_3 exposures, though significant changes were found after O_3 exposure for 1 or 3 h for 5 consecutive days ($F = 248.1$, df = 3, $p < 0.05$) (Figure 2A). Significant changes in IL-6 levels were found in the cerebral cortex at exposures of 1, 3 or 6 h ($F = 9.474$, df = 3, $p < 0.05$) or after daily exposures of 3 h for 5 consecutive days ($F = 6.503$, df = 2, $p = 0.05$) (Figure 2B).

Neuroinflammation was evidenced by significant increases in cells immunopositive for NF-κB p50 after 3 or 6 h of O_3 exposure ($F = 62.48$, df = 3, $p < 0.0001$) and after 1 or 3 h of O_3 exposure for 5 consecutive days ($F = 163.9$, df = 2, $p < 0.0001$) (Figure 3). We also found significant increases in GFAP levels in the cerebral cortex following 6 h of O_3 exposure ($F = 5.758$, df = 3, $p < 0.05$) and following O_3 exposure for 1 or 3 h for 5 consecutive days ($F = 54.76$, df = 2, $p < 0.05$) (Figure 4).

Discussion

Activation of the immune system induces the overexpression of macrophages, neutrophils and pro-inflammatory cytokines such as TNF-α and IL-1β, which in turn induce the expression of other cytokines, e.g. the IL-6, IL-8 and IL-4 (Nichols et al., 2001; Tanabe et al., 2010). The increases in the above cytokines depend directly on the phosphorylation of transcriptional factors such as NF-κB (Cho et al., 2007; Pahl, 1999; Yamaguchi et al., 2009). Consistent with this cytokine activation pattern, in the present study, we tested the expression of TNF-α, IL-6 and NF-κB after O_3 exposure, in both the lung and the cerebral cortex.

Bronchoalveolar lavages obtained from healthy volunteers exposed to 0.20 ppm of O_3 during 4 h shows a significant increase in neutrophils (Aris et al., 1993). Exposure of human bronchial epithelial cells (BEAS-2B cells) to 0.08 ppm (0.16 mg/m^3) induces the overexpression of IL-1 and IL-6 (Song et al., 2011). Macrophages obtained from BALF of healthy volunteers exposed to 0.40 ppm of O_3 during 1 h show significant increases in TNF-α, IL-1β, IL-6 and IL-8, whereas macrophages from BALF of guinea pigs submitted to the same

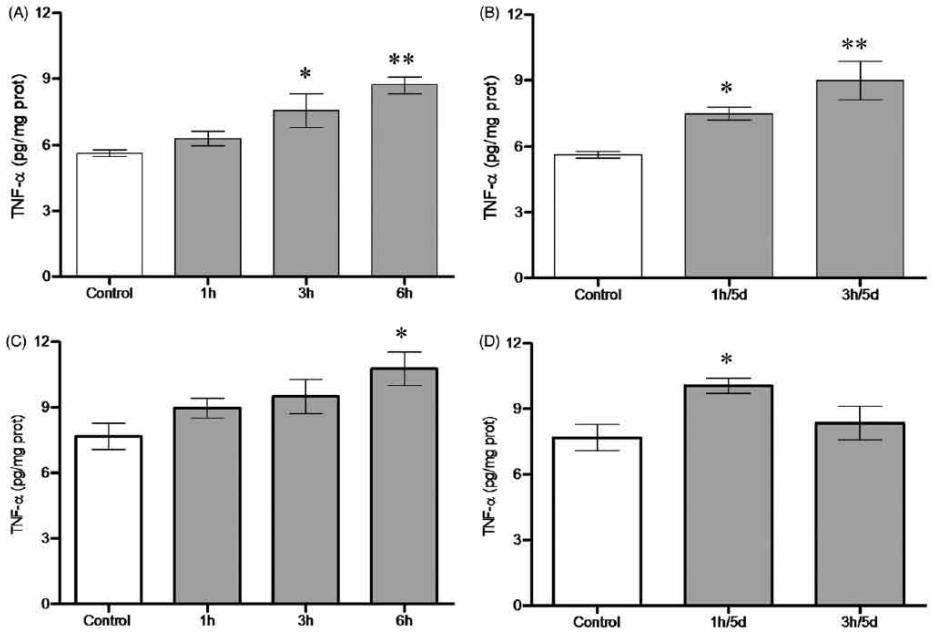

Figure 1. The TNF-α concentration after O_3 exposure, detected using ELISA. (A) A graph showing the significant increases in TNF-α from lung after 3 and 6 h of O_3 exposure and after exposure for 1 and 3 h/d for 5 consecutive days. (B) A graph showing the significant increases in TNF-α from cerebral cortex after 6 h of O_3 exposure and after 1 h daily exposure for 5 consecutive days. *$p = 0.05$; **$p = 0.01$.

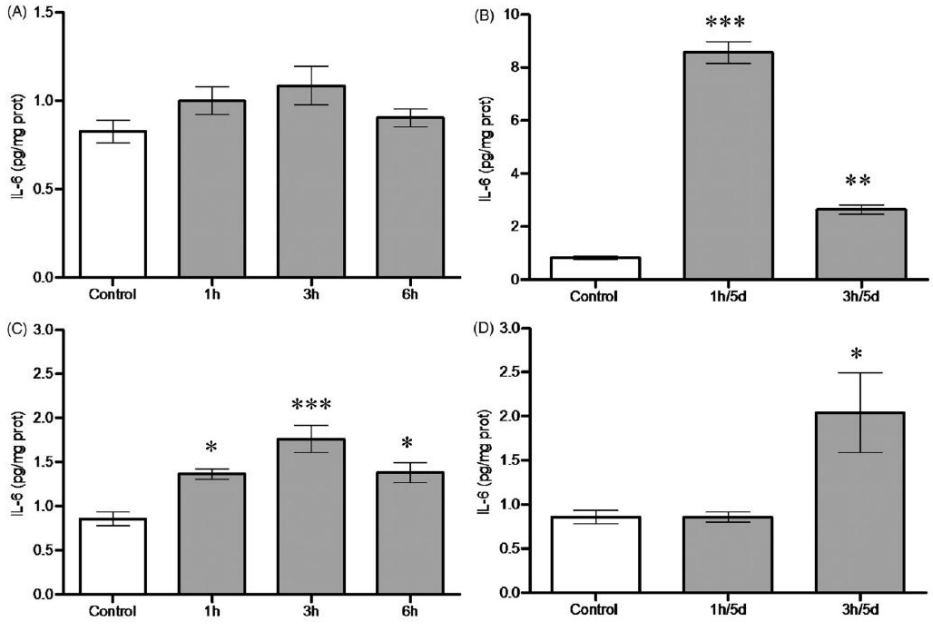

Figure 2. The concentration of IL-6 following O_3 exposure, detected using ELISA. (A) A graph showing the significant increases in IL-6 from lung after 1 and 3 h daily exposure for 5 consecutive days. (B) A graph showing the significant increases in IL-6 from cerebral cortex following 1, 3 or 6 h of O_3 exposure and after 3 h daily exposure for 5 consecutive days. *$p = 0.05$; **$p = 0.01$; ***$p = 0.001$.

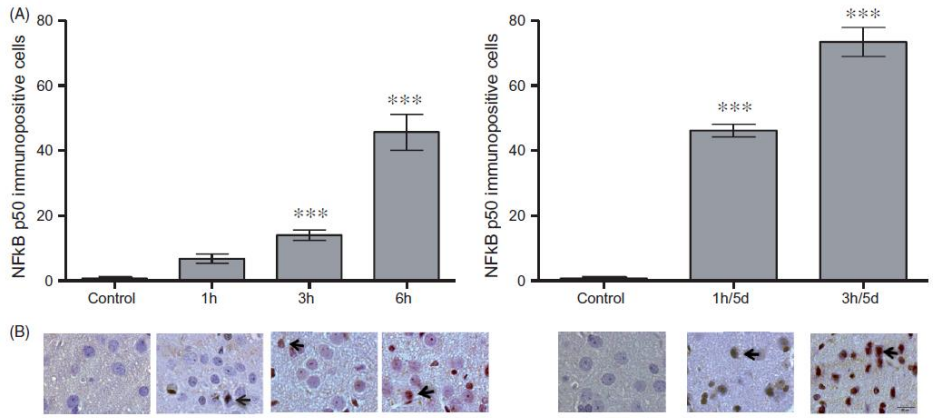

Figure 3. Cells immunopositive for NF-κB p50 in cerebral cortex of rats exposed to O_3. (A) A significant increase in the number of cells immunopositive for NF-κB p50 after 3 and 6 h of O_3 exposure and after 1 or 3 h daily exposure for 5 consecutive days. (B) Representative micrographs (60×) of cells immunopositive for NF-κB p50 in sagittal sections of right cerebral cortex after O_3 exposure. Representative immunopositive cells for NF-κB p50 are showed with a black arrow. ***$p = 0.001$.

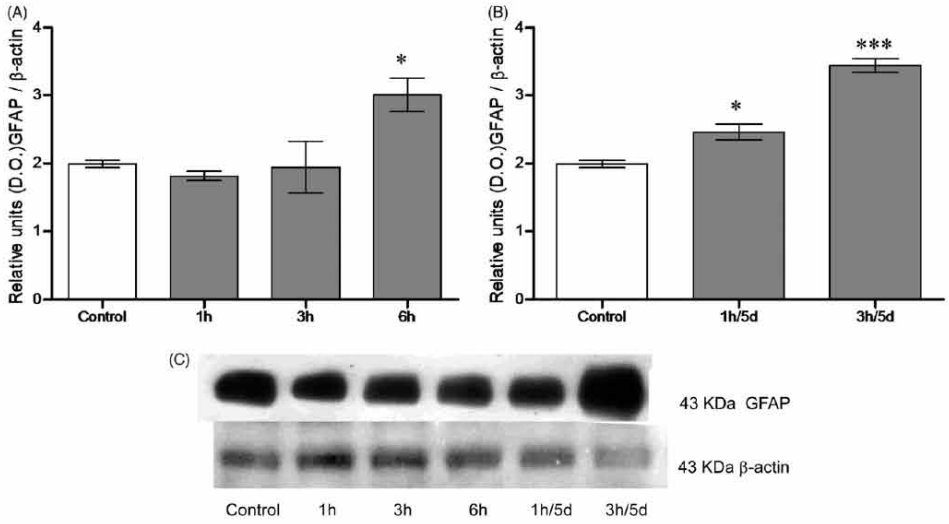

Figure 4. Determination of GFAP levels in the cerebral cortex of rats exposed to O_3, detected by western blotting analyses. (A) A graph showing a significant increase in protein expression after 6 h of O_3 exposure and after 1 or 3 h exposure per day for 5 consecutive days. (B) A representative blot showing the protein quantification as determined by optical densitometry; β-actin was used as a control. $^*p = 0.05$; $^{***}p = 0.001$.

O_3 conditions show significant increases in TNF-α and IL-6 (Arsalane et al., 1995). Moreover, BALF of mice exposed to 0.3 ppm O_3 over 48 h shows a significant increase in neutrophils, macrophages, lymphocytes and TNF-R1 (Cho et al., 2001). Epithelial cells and macrophages obtained from BALF of rats exposed to 3.00 ppm of O_3 for 6 h show significant increases in the expression of a chemokine (CINC-1), NF-κB and IL-1β (Haddad et al., 1996; Manzer et al., 2008). Such lung inflammation products induced by O_3 exposure have been associated with lung function impairment, such as exacerbation of asthma, and airway damage, according to the US Environmental Protection Agency.

As most studies on O_3 exposure have focused on respiratory tract inflammation, less is known about the mechanisms that could explain the brain dysfunction induced by O_3 exposure. However, some neurological manifestations, such as lethargy, fatigue and headache have been reported by inhabitants of polluted areas with high O_3 concentrations (Dales et al., 2009; Winquist et al., 2012). Experimental studies in different murine strains show that exposure to 0.60–1.00 ppm O_3 decreases motor activity (Dorado-Martinez et al., 2001; Rivas-Arancibia et al., 2003; Tepper et al., 1982), induces memory impairment (Avila-Costa et al., 1999) and causes deficiencies in social behavior (Sorace et al., 2001). A significant decrease of REM sleep and increase in slow-wave sleep has been identified in electrographic studies recorded in rats and cats after O_3 exposure (Arito et al., 1992; Paz & Bazán-Perkins, 1992). These changes in sleep induced by O_3 exposure may be due to changes in the concentration of neurotransmitters in brain regions related to sleep regulation. Neurochemical analyses demonstrate that exposure to 1.00–1.50 ppm of O_3 increases the concentration and metabolism of 5-HT, as well as the levels of dopamine and noradrenaline in the rat brainstem (González-Piña & Paz, 1997; Huitrón-Reséndiz et al., 1994; Paz & Huitrón-Reséndiz, 1996) and inhibits the brainstem activity of tyrosine hydroxylase, the limiting enzyme in catecholamine biosynthesis after 0.50 ppm O_3 exposure for 5 d (Cottet-Emard et al., 1997).

The disruption of sleep patterns due to O_3 exposure can be reversed by the use of an anti-inflammatory such as indomethacin (Rubio & Paz, 2003). To characterize the possible inflammatory mechanisms through which O_3 affects tissues beyond the pulmonary system, we evaluated the early inflammatory response in the cerebral cortex. In lung, we found a significant increase in TNF-α after the 3 h exposure to O_3, and this response is observed after 1 or 3 h of daily exposure for 5 d. Furthermore, an increased concentration of IL-6 was found in lung after 1 or 3 h O_3 exposure for 5 d. These results are consistent with previous reports from airways of humans and animals exposed to O_3 (Devlin et al., 1996; Cho et al., 2007). In cerebral cortex, we found a significant increase in the TNF-α level after a single 6 h exposure to O_3 and in rats exposed to this gas for 1 h/d for 5 consecutive days. Furthermore, a significant increase of IL-6 was found after O_3 exposure for 1, 3 or 6 h or for 3 h/d for 5 consecutive days. After 1 h of O_3 exposure we did not find significant changes in TNF-α at both the lung and the cerebral cortex, as well as in IL-6 at the lung, probably because the rats were sacrificed immediately after the exposure. However, TNF-α and IL-6 increase significantly after several days of O_3 exposure during 1 h, probably by a cumulative effect of such cytokines. We found that TNF-α and IL-6 showed a tendency to increase after a sole exposition to O_3 at the lung and the cerebral cortex, indicating that such inflammatory response occurred simultaneously after the same toxicological stimulation. However, there is an uncoupling of data for the lung

and the cerebral cortex of the TNF-α and IL-6 levels after 1 or 3 h of O_3 exposure for 5 consecutive days. Such differences could prove that the acute inflammation and chronic inflammation are different types of adaptive response that are called into action when other homeostatic mechanisms are either insufficient or not competent; or due to the intrinsic pleiotropic effects of those cytokines (Medzhitov, 2008; Tarrant, 2010).

It is known that both TNF-α and IL-6 can cross the blood–brain barrier to reach the CNS by simple diffusion or by means of specific transporters located in the endothelial cells of the blood–brain barrier (Banks, 2005). Also, the O_3 inhalation induces metaplasia and infiltration of neutrophils in the nasal epithelium. The significant increases of mucosal neutrophils produce an inflammatory response directly by the injury to nasal cavity (Harkema & Wanger, 2005); these epithelial and endothelial cells in the cavernous sinus release cytokines that can directly increase both the systemic and neuroinflammatory response (Genc et al., 2012). In CNS, those cytokines are capable of stimulating the release of IκBα from NF-κB, which translocates the subunits NF-κB p50 and NF-κB p65 to the nucleus to activate the expression of multiple genes implicated in the inflammatory response (Cho et al., 2007; Pahl, 1999; Stein et al., 1993). We found significant increases in NF-κB p50 after single O_3 exposures of 1, 3 and 6 h, as well as after 1 and 3 h exposures for 5 consecutive days. NF-κB p50 is also capable of inducing the expression of GFAP (Sticozzi et al., 2013). GFAP then induces the expression of several genes implicated in the inflammatory response (Okada et al., 2006). Our results showed that GFAP expression increased after 6 h of exposure or after 1 or 3 h of exposure for 5 consecutive days. Furthermore, it has been demonstrated that both TNF-α and IL-6 administered intracerebroventricularly can induce significant increases in SWS and decreases in REM (Krueger, 2009; Dantzer & Kelley, 2007). Increased SWS and decreased REM have been previously shown in cats and rats after O_3 exposure at different concentrations (Arito et al., 1992; Huitrón-Reséndiz et al., 1994; Paz & Bazán-Perkins, 1992; Paz & Huitrón-Reséndiz, 1996). Therefore, we conclude that the presence of cytokines in the brain, as described in the present study, could explain the mechanism by which O_3 induces the neurological alterations reported after O_3 exposure.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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The effects of ozone exposure and associated injury mechanisms on the central nervous system

Abstract: Ozone (O_3) is a component of photochemical smog, which is a major air pollutant and demonstrates properties that are harmful to health because of the toxic properties that are inherent to its powerful oxidizing capabilities. Environmental O_3 exposure is associated with many symptoms related to respiratory disorders, which include loss of lung function, exacerbation of asthma, airway damage, and lung inflammation. The effects of O_3 are not restricted to the respiratory system or function – adverse effects within the central nervous system (CNS) such as decreased cognitive response, decrease in motor activity, headaches, disturbances in the sleep-wake cycle, neuronal dysfunctions, cell degeneration, and neurochemical alterations have also been described; furthermore, it has also been proposed that O_3 could have epigenetic effects. O_3 exposure induces the reactive chemical species in the lungs, but the short half-life of these chemical species has led some authors to attribute the injurious mechanisms observed within the lungs to inflammatory processes. However, the damage to the CNS induced by O_3 exposure is not well understood. In this review, the basic mechanisms of inflammation and activation of the immune system by O_3 exposure are described and the potential mechanisms of damage, which include neuroinflammation and oxidative stress, and the signs and symptoms of disturbances within the CNS caused by environmental O_3 exposure are discussed.

Keywords: air pollution; central nervous system; inflammation; oxidative stress; ozone.

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Introduction: systemic consequences of ozone exposure on health

Atmospheric pollution represents a serious health problem, particularly in developing countries where millions of people are chronically exposed to air pollutants (Paz, 1997; Block and Calderon, 2009). Ozone (O_3), particulate matter (PM), and various biological materials are the main air pollutants that we breathe and that can cause severe health damage. It is estimated that air pollution is the eighth highest mortality risk factor, accounting for 2.5% of all deaths in developed countries (Narayan et al., 2010). Additionally, an increase in the admission of people into hospitals, which is reflected by loss in labor productivity, because of O_3 pollution has been reported (Jörres et al., 1996; Frank et al., 2001; Szyszkowicz et al., 2009).

O_3 can be naturally generated by the photodissociation of ultraviolet rays from the sun at low wavelengths on oxygen molecules (O_2) in the lower atmosphere. O_3 can also be formed by high-voltage discharges from engine friction, neon light signs, and other electrical equipment such as xerographic copiers, electrostatic air cleaners, printers, and workplaces where welding is used. Moreover, O_3 is generated and used in the purification of air systems in buildings, in the control of fungal and bacterial growth in cold storage plants, in the treatment of residual waters, and in the purification of drinking water.

The World Health Organization (WHO, 2005), in its document WHO Air Quality Guidelines for Particulate Matter, Ozone, Nitrogen Dioxide and Sulfur Dioxide, established the permissible limits of O_3 exposure at an average of 120 μg/m³ (60 ppb) for a maximum of 8-h concentration. However, diminished pulmonary function

has been observed in children exposed to low concentrations of such gases as well as in some individuals who are intrinsically sensitive to those gases (Jörres et al., 1996). At present, there are many urban areas that have been measured and showed O_3 concentration >120 μg/m³ (60 ppb/8 h). The absorption average of inhaled O_3 in humans is between 40% and 65% during repose states (Gerrity et al., 1988; Kabel et al., 1994); however, only a small fraction (4–6%) of the total O_3 dose can react with cellular membranes (Freeman and Mudd, 1981; Pryor, 1992) to trigger a cascade of reactions (Pryor et al., 1995b) that result in the formation of reactive oxygen species (ROS) (Kennedy et al., 1992; Pryor et al., 2006), accumulation of oxidized biomolecules (Pryor et al., 1991), and the activation of inflammatory processes in epithelial cells (Pryor et al., 1995a). The symptoms associated with O_3 exposure are related to the airways, which include loss in lung function, exacerbation of asthma, airway damage, and lung inflammation [Lippmann, 1993; US Environmental Protection Agency (US EPA), 1996]. Reports of the transmigration of macrophages, neutrophils, and other immune cells in volunteer subjects after a controlled exposure, an increase of neutrophils in nasal washes (Graham et al., 1988), and the atrophy of nasal cilia and basal cell hyperplasia of residents near the most polluted areas of cities have been reported (Calderon-Garciduenas et al., 1998). Nevertheless, it cannot be excluded that O_3 acts as a pro-oxidant for a complex mixture of combustion pollutants that originates primarily from vehicular traffic, which increases in the summer due to geographic and climatic factors (Latzin et al., 2009).

The effects of O_3 overexposure initiate a rapid damage to the bronchial-alveolar epithelium (Postlethwait et al., 2000), which increases its permeability and induces inflammation. These cellular changes increase the release of mediators and chemotactic factors, which results in edema, pulmonary emphysema, and fibrosis (Seltzer et al., 1986). It has been proposed that during this acute phase of exposure, cellular necrosis predominates due to direct cytotoxicity from the generation of free radicals (FR). FR can be ROS or reactive nitrogen species (RNS), which exacerbate the damage in the body (Pryor and Church, 1991; Dorado-Martinez et al., 2001). The increased production of ROS and RNS triggers the response of antioxidant systems to counteract lipid peroxidation and minimizes cell damage; however, when the balance between ROS generation and antioxidant systems breaks, the cells reach a state of oxidative stress (Sies, 1991).

Oxidative stress produces cellular damage by an increase in the expression of the inducible nitric oxide synthase (iNOS) (Laskin et al., 2002). iNOS produces nitric oxide (NO), which activates guanylate cyclase to generate guanosine 3′,5′-cyclic phosphoric acid (cGMP), which, in turn, enhances the concentration of intracellular Ca^{2+} and subsequently activates phospholipase A2 (PLA2), C (PLC), and D (PLD) (Wright et al., 1994; Kafoury et al., 1998). The phospholipids of the cell membrane and diacylglycerols are hydrolyzed by phospholipases, producing arachidonic acid (AA) and inositol triphosphate (IP3). AA is a substrate for cyclooxygenase (COX) and/or lipoxygenase (LOX); COX produces prostaglandin E2 (PGE_2), which activates adenylate cyclase. This induces the production of adenosine-3′,5′-monophosphate (cAMP), which activates protein kinase A and the release of corticosteroids (Ignarro, 1991; Mohn et al., 2005). In addition, AA is a precursor of the isoprostanoid 8-iso-prostaglandin F2a, which is derived from β-oxidation (Chiabrando et al., 1999) and released into the circulatory system in response to O_3 exposure (Montuschi et al., 2002). Meanwhile, LOX activity produces leukotrienes. An increase in leukotrienes and prostaglandins produces ROS, lipid hydroperoxides, interleukin (IL) 6 (IL-6), IL-8, and other cytokines, which are involved in the inflammatory response (Devlin et al., 1994; Kafoury et al., 1999) (Figure 1).

O_3-exposure studies have reported increased levels of pro-inflammatory markers (cytokines or chemokines) in bronchial lavage fluid. These studies report an increased expression of tumor necrosis factor (TNF), IL-1, IL-2, IL-6, IL-8, cytokine-induced neutrophil chemoattractant 1 as well as chemokines such as monocytes 1 chemoattractant protein (Haddad et al., 1996; Manzer et al., 2008). All of these pro-inflammatory factors are increased *via* the activation of nuclear factor κB (NFκB) (Haddad et al., 1996). The NFκB pathway can be directly activated by the cytokines IL-1 and TNF-β, which amplifies the inflammatory response and maintains the persistence of chronic inflammation in local sites.

NFκB can also stimulate the expression of enzymes such as iNOS and COX-2 (Pahl, 1999). iNOS produces NO, which significantly participates in nearly all of the developmental stages of inflammation, particularly in the regulation of endothelial inflammation and in the early stages of the transmigration of inflammatory cells into inflammatory sites. NFκB also increases the expression of cytokines, chemokines, the major histocompatibility complex (MHC) as well as recipient neutrophil adhesion and migration (Haddad et al., 1996; Ghosh et al., 1998; Pahl, 1999). Once these products are generated, they can migrate freely in the bloodstream to other organs. In this way, O_3 can induce damage in tissues that are localized at

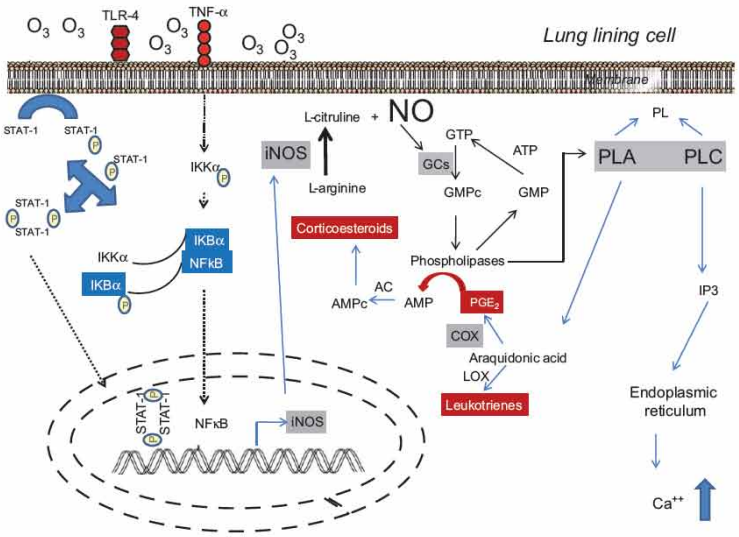

Figure 1 Biochemical and molecular effects of O_3 exposure on the epithelial cells of the upper and lower airways.
O_3 exposure may induce the activation of TLRs and the TNF-α receptor. TLR4 activation induces the phosphorylation of the signal transducer and activator of transcription 1, TNF-α induces the activation of IKKα, which phosphorylates IKBα and releases the NFκB. Both STAT1 and NFκB in the nucleus increase the expression of iNOS. The increased synthesis of NO activates soluble guanylate cyclase, which converts GTP *via* the cGMP phospholipases PLA2 and PLC. Both phospholipases act on the membrane phospholipids by releasing AA and IP3 in PLA2 and PLC, respectively. AA serves as a substrate for LOX and COX. LOX increases the levels of leukotrienes and COX can generate PGE2, which activates adenylate cyclase, thereby increasing cAMP levels and the subsequent release of corticosteroids. IP3 induces the release of Ca^{2+} in the endoplasmic reticulum.

sites distant from the area of exposure, even in the central nervous system (CNS).

O_3 inhalation induces the activation of the immune system, which is mediated by several factors and cells that regulate immune response. O_3 increases the amount of phagocytic cells (macrophages) and neutrophils in the airways (Devlin et al., 1994; Kafoury et al., 1999; Neuhaus-Steinmetz et al., 2000), increases the levels of the platelet-activating factor (Wright et al., 1994), TNF (Cho et al., 2007), and decreases the response of C fibers (Joad et al., 1996, 2000). O_3 also increases the production of γinterferon (Bocci et al., 1998), which increases the production of macrophages (Wiester et al., 1996b). O_3 stimulates leukocytic production, which can induce allergic reactions (Neuhaus-Steinmetz et al., 2000). It can stimulate the production of IL-2, which is required for the production of T lymphocytes (Song et al., 2011), as well as induce the production of antioxidant enzymes (Gomez-Mejiba et al., 2009).

O_3 inhalation can activate the IL-1 receptor (Park et al., 2004) and the Toll-like 4 receptor (TLR4), which are mainly activated by endotoxins (Schuster and Nelson, 2000; Hollingsworth et al., 2007). The activation of these two receptors results in the activation of ubiquitous and pleiotropic transcription factors such as NFκB (Schuster and Nelson, 2000) and erythroid ratio factor 2 (Nrf2), which significantly contribute to the expression of genes involved in the enzymatic antioxidant response [superoxide dismutase (SOD), catalase, and glutathione peroxidase] and cellular detoxification of ROS produced by atmospheric pollution. O_3 exposure results in the dissociation of Nrf2 from Keap1, which enables Nrf2 to translocate into the nucleus where it dimerizes with Maf proteins, resulting in the binding of Nrf2 to the antioxidant response elements, inflammatory molecules, and immune response suppressors (Osburn and Kensler, 2008; Cho and Kleeberger, 2010; Rubio et al., 2010). The CNS immunological response is regulated by humoral mechanisms, including IL-10 (Backus et al., 2010), norepinephrine (Hu et al., 1991), and α melanocyte-stimulating hormone (Lipton and Catania, 1997).

Some consequences of O_3 exposure on the CNS

The entry and exit of substances into the CNS is determined by the blood-brain barrier (BBB). This barrier helps regulate the innate immune response as well as the recruitment and entry of leukocytes and are thus involved in both the surveillance and the reactive functions of the central immune cell population (Abbott and Friedman, 2012). In 1885, Paul Ehrlich examined the restricted permeability of the BBB and described that an intravenous injection of aniline could dye the entire body except for the brain, which determined that the integrity of endothelial cells as an essential prerequisite for CNS health. Under normal conditions, cytokines secreted by the immune system in the periphery cannot cross the BBB; however, there is evidence that the circulating molecules produced by acute and chronic O_3 exposure can cross to the brain *via* three potential entrances: (a) damaged areas in the BBB (Calderon-Garciduenas et al., 2002), (b) choroid plexus (Block and Calderon, 2009), and (c) circumventricular organs (Banks, 2005). However, the active transport of these molecules by transporters expressed in the endothelial cells adjacent to the BBB is also possible (Abbott and Friedman, 2012). Adjacent cells produce cytokines *de novo*, which supports the production of soluble factors that activate neurons directly or indirectly through astroglial (microglia and astrocytes) activation (Turrin and Rivest, 2004). Although the mechanism by which astroglia are activated in the brain remains unclear, it is known that astroglia are sensitive to mechanisms of inflammation and oxidative stress derived from damage induced by air pollution in other cell types (Block and Calderon, 2009). Astrocytes express TLRs, which, when activated, can produce various inflammatory mediators, including cytokines, which can amplify the local immune response and modify BBB permeability (Brambilla et al., 2005; Bsibsi et al., 2006). Changes associated with the loss of BBB integrity and leukocyte infiltration followed by red cell entry into the brain have been demonstrated in subjects exposed to atmospheric pollutants, including O_3 (Calderon-Garciduenas et al., 2008). Accordingly, a compromise in BBB function accompanies many neurological disorders and is closely associated with brain inflammatory processes initiated by both leukocytes infiltration from the blood and activation of glial cells. Those inflammatory processes contribute to the severity and prognosis of numerous neurological disorders and can both be the cause and the result of BBB dysfunction (de Vries et al., 2012). Evidence was linked to the increase in the concentration of ROS and RNS and metalloproteinase activation, which induce the degradation of tight junctions, leading to BBB breakdown (Thiel and Audus, 2001; Gu et al., 2011). Experimental studies of O_3 exposure *in vivo* showed the presence of astrocytes with increased expression of IL-6 and TNF-α near the periphery of the brain capillaries (Araneda et al., 2008). In addition, astrocytic cultures exposed to O_3 showed increased cell death (Zhou et al., 2008) (Figure 2).

The current consensus is that pro-inflammatory cytokines, particularly IL-1β, TNF-α, and IL-6 generated in the periphery, are directed into the CNS where they are able to induce the synthesis of additional cytokines and other pro-inflammatory molecules (Ek et al., 1998; Bluthe et al., 2000a,b). Bronchial C-fibers and adapting receptors appear to be the primary vagal afferents responsible for O_3-induced changes in ventilatory rate in both humans (Folinsbee and Hazucha, 2000; Schelegle et al., 2001) and animals (Schelegle et al., 1993). The stimulation of vagal afferents by O_3 and reactive products is enhanced and sustained by secondary mechanisms activated at cellular and molecular levels (Schelegle et al., 1993). The activation of vagal afferents sensitizes the CNS to the presence of peripheral inflammatory molecules, which results in the activation of the hypothalamic-pituitary-adrenal (HPA) axis, thus inducing an anti-inflammatory response (Pavlov and Tracey, 2006). Cytokines regulate the feedback loop between glucocorticoids and the HPA axis. Several neuronal circuits related to physiological processes such as thermoregulation, food intake, and sleep patterns are also regulated by this axis. The relationship between the immune system and the nervous system is regulated at different levels, although the main communication, which occurs between the CNS and the immune system, is *via* the HPA axis (Dantzer et al., 2000; Besedovsky and Rey, 2007). The first reported cytokine described for its ability to activate the axis HPA was IL-1β; however, the activation of this axis may also be triggered by other cytokines (Besedovsky et al., 1986) that increase with O_3 exposure (Wu et al., 2008). This activation in the brain causes the release of corticosteroids in the blood. These hormones not only mobilize energy reserves but they also reduce the number and effector functions of lymphocytes, which generates immunosuppressive effects (Franchimont, 2004).

Studies of controlled exposure to different doses of O_3 in humans report specific alterations in CNS function, including lethargy (Hackney et al., 1975), fatigue and cephalalgy (Hackney et al., 1977), and memory impairment (Lategola et al., 1980). Studies in experimental models describe a decrease in motor activity (Tepper et al., 1982; Dorado-Martinez et al., 2001; Rivas-Arancibia et al., 2003) and alterations in the sleep-wake cycle

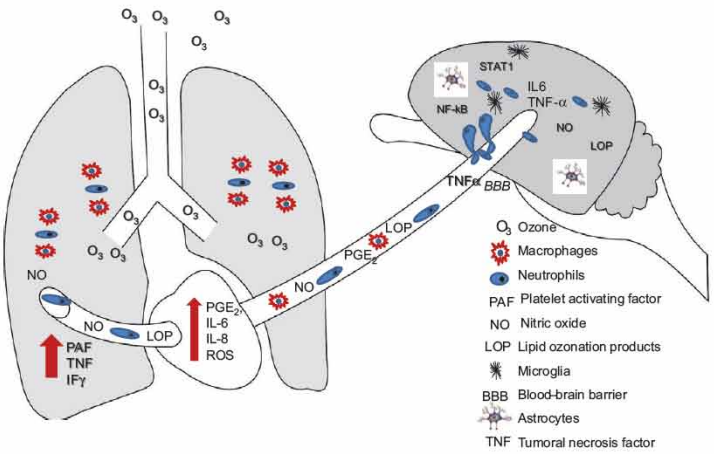

Figure 2 Activation of the microglia and astrocytes by O_3 exposure.
Molecules from acute and chronic O_3 exposure can enter the circulation to the brain *via* three potential entry routes: damaged areas of the BBB, choroid plexus, and circumventricular organs. In addition to being actively transported by transporters expressed in adjacent endothelial cells, cells adjacent to the BBB also generate cytokines that favor the production of soluble factors and directly activate neurons or microglia and astrocytes.

(Paz and Bazan-Perkins, 1992; Huitron-Resendiz et al., 1994). Studies in experimental animals have enabled the exploration of the consequences of O_3 exposure in specific regions of the CNS (Paz, 1997). These results show a dose-dependent increase in the presence of oxidative stress markers in regions such as the frontal cortex, hippocampus, striatum, cerebellum, and olfactory bulbs. These regions exhibit mitochondrial edema, defects in the endoplasmic reticulum, and alterations in overall cell function. From the molecular perspective, these alterations may explain changes such as increasing levels of lipid ozonation products (Dorado-Martinez et al., 2001; Rivas-Arancibia et al., 2003) and alteration in neurotransmitter (NT) concentrations such as γ-amino butyric acid (GABA) (Rivas-Arancibia et al., 2003), glutamate (Santiago-Lopez et al., 2010), serotonin (5-HT) (Huitron-Resendiz et al., 1994; Paz and Huitron-Resendiz, 1996), noradrenaline (NA) (Gonzalez-Pina et al., 2008; Custodio et al., 2010), and NO (Dorado-Martinez et al., 2001). Changes in the synthesis and degradation of NT enzymes, for example, the inhibition of the tyrosine hydroxylase activity after O_3 exposure (Cottet-Emard et al., 1997), have also been reported. Furthermore, changes in NT levels also correlate with alterations in normal neurophysiological processes.

O_3 and sleep

Sleep is a naturally reversible functional state that is characterized by a reduction in voluntary motor activity, an increase in the threshold to external stimuli response, and stereotypical posture. It has been suggested that sleep is a highly regulated phenomenon because it has different levels of biological regulation including genetic and synaptic control as well modulation based on the interaction of neuronal networks. On the basis of mainly electrophysiological characteristics, normal sleep can be divided into two states, rapid eye movement (REM) and non-rapid eye movement (NREM) sleep, that cyclically alternate throughout a sleep episode (O'Donnell et al., 1971; Groll-Knapp et al., 1982; Fraigne et al., 2008). Recently, some models have suggested that sleep is modulated by flip-flop switches that are characterized by neuronal circuits with different NTs and that interact to regulate the initiation and maintenance of the different stages of the sleep-wake cycle. Therefore, within the brainstem, basal forebrain, and hypothalamus, there are a number of neuronal populations that promote wakefulness through the action of different NTs such as acetylcholine, NA, 5-HT, histamine, and orexin. Meanwhile, neurons located in the hypothalamus and brainstem are involved in initiating

and maintaining sleep. These neurons contain NTs such as acetylcholine and GABA, which have projections to the nuclei involved in wakefulness regulation (Franco-Pérez et al., 2012).

Currently, there are few reports on the effects of different air pollutants on sleep architecture in humans and experimental animal models. Studies on the effects of O_3 exposure on sleep began in the early nineties, when research groups in Mexico and Japan described the specific effects of O_3 exposure on circadian phenomena such as the sleep-wake cycle. Using rats as an experimental model, Arito et al. (1992) found that exposure to 0.5 and 1 ppm of O_3 suppressed wakefulness and REM at the expense of an increase in NREM. In addition, Paz and Bazan-Perkins (1992) reported that exposure to 1.2 ppm of O_3 caused an increase in NREM and a decrease of REM in cat sleep pattern but no alteration in wakefulness. Subsequently, this same group conducted a series of experiments in rats in which the exposure times and concentrations of O_3 varied. They found the same effects that have been previously described in cats, along with an increase in NREM and decrease in REM. However, only high doses (1.5 ppm) of O_3 caused a decrease in wakefulness (Huitron-Resendiz et al., 1994; Paz and Huitron-Resendiz, 1996) (Table 1).

The effects of O_3 exposure on sleep may be due to changes in the concentrations of various NT in different brain regions. For example, neurochemical analyses showed that exposure to 1 and 1.5 ppm of O_3 increased the concentration and metabolism of 5-HT as well as the levels of dopamine and NA in the brainstem of rats (Huitron-Resendiz et al., 1994; Paz and Huitron-Resendiz, 1996; Gonzalez-Pina and Paz, 1997). Similarly, another study demonstrated that the concentrations of extracellular acetylcholine dramatically decreases in the hypothalamus of adult rats exposed to 0.5 ppm (Alfaro-Rodriguez and Gonzalez-Pina, 2005). Although the determination of NT concentrations in specific brain regions may partially explain the decrease of REMs caused by O_3 exposure, there are currently no studies that demonstrate an increase in NREM with varying concentrations of NT. However, this phenomenon has been approached from another perspective. Several molecules such as peptides, lipids, and even cytokines exhibit hypnogenic properties and are referred to as 'sleep-inducing factors'. Some cytokines such as IL-1 and TNF have been demonstrated to regulate the expression of COX and increase the total time spent in NREM (Garcia-Garcia et al., 2009). Pretreatment with indomethacin, a nonsteroidal anti-inflammatory and COX inhibitor, reduces the increase in NREM observed after O_3 exposure (Rubio and Paz, 2003). In addition, central or systemic administration of IL-1 or TNF increased the duration of NREM sleep and the power spectrum of δ waves, as assessed by an EEG, which is an indicator parameter of the intensity of NREM sleep (Shoham et al., 1987). All of these data suggest that inflammatory factors may mediate the increase in NREM caused by O_3 exposure.

Sleep is a process associated with specific functions such as energy conservation, immune function, brain metabolism, neural network maintenance, learning, memory, and brain plasticity. Thus, it is very important to consider the changes in sleep patterns caused by O_3 exposure because this phenomenon may alter proper brain function.

Teratogenic effects of O_3 exposure

Exposure to toxic air agents in polluted urban areas can interfere with prenatal and postnatal development. Despite the differences in the sequences and processes of brain development, which vary from one species to another, and the differences among the various brain

Table 1 Experimental evidence of the abnormal sleep-wake cycle caused by O_3 exposure.

Concentration of O_3 (ppm)	Exposition time (h)	Wakefulness	NREM	REM	Species and reference
0.35	24	=	=	↓	Rat (Paz and Huitron-Resendiz, 1996)
0.40	24	=	=	=	Cat (Paz and Bazan-Perkins, 1992)
0.50	6	↓	↑	↓	Rat (Arito et al., 1992)
0.75	24	=	↑	↓	Rat (Paz and Huitron-Resendiz, 1996)
0.80	24	=	↑	=	Cat (Paz and Bazan-Perkins, 1992)
1.00	3	↓	↑	↓	Rat (Arito et al., 1992)
1.20	24	=	↑	↓	Cat (Paz and Bazan-Perkins, 1992)
1.50	24	↓	↑	↓	Rat (Huitron-Resendiz et al., 1994)
1.50	24	↓	↑	↓	Rat (Paz and Huitron-Resendiz, 1996)

Effect of exposure to different concentrations of O_3 on sleep parameters: = no changes, ↓ decrement, ↑ increment.

structures, a vulnerable period during which exogenous agents in the environment can cause alterations in the development of the CNS has been proposed. During this period, the CNS is more susceptible to neurotoxic agents compared with the adult brain (Thombur and Moore, 1976).

The effects of O_3 exposure during gestation begin during implantation. Reports show a decrease in the number of implantations, increases in embryo reabsorption *in utero*, decreases in fetal weight, skeletal ossification, and reduction of neonatal development in offspring (Kavlock et al., 1979, 1980). Moreover, there have been several reports on the significant effects of reproductive patterns, somatic and neurobehavioral development, and motor behavior of the offspring (Petruzzi et al., 1995), decreased litter size, and increased neonatal death (Veninga, 1967). However, these reports are not conclusive, as there have been other reports indicating that prenatal O_3 exposure does not significantly affect the length of gestation, litter size, sex ratio, or neonatal mortality (Bignami et al., 1994).

One factor involved in the deterioration of the developing fetus is the effect of air pollution on the changes in the functional morphology of the placenta. The morphology of the placenta of mice exposed to unfiltered ambient air (experimental group) showed a reduction in the caliber and maternal blood spaces compared with the placenta from the control group (filtered air). Thus, urban air pollution affects the functional morphology of the placenta, which is the main link between the mother and the fetus (Veras et al., 2008).

It has been reported that the concentration of NA in the cerebellum is significantly lower because of O_3 exposure during pregnancy, and this effect is maintained throughout the different stages of postnatal cerebellar development (birth, 10, 20, and 30 days) (Custodio et al., 2010). These results may be related to the morphological changes previously described by Rivas-Manzano and Paz (1999). The maturation of the mammalian brain occurs *in utero*; however, there are many events that occur in the postnatal period, such as glial proliferation, myelination, dendritic development, and postnatal neurogenesis (Watson et al., 2006). Cerebellar morphological studies in rats exposed to 1 ppm of O_3 during gestation showed significant decreases in the anterior lobe compared with rats that were not exposed. In addition, there were signs of necrosis in the Purkinje cells of newborn rats. This damage was permanent at 12 and 60 days of postnatal O_3 exposure during gestation, which induced a permanent cerebellar damage in the rats (Rivas-Manzano and Paz, 1999). These pathological findings may be related to the high production of FR induced by O_3 exposure during pregnancy.

Exposure to environmental factors during gestation may also trigger inflammatory processes that cause brain damage to progeny (Zanchi, 2012). The systemic inflammation produced by O_3 exposure during prenatal development can induce changes in the cytokine concentration and other pro-inflammatory molecules, such as what occurs in damages caused by endotoxins, which result in cerebral hypoperfusion and the activation of apoptosis in oligodendrocyte progenitor cells *via* the release of pro-inflammatory cytokines (Berger et al., 2002). IL-1β stimulates the growth of astrocytes and increases GFAP expression in reactive astrocytes. These observations suggest a potential role of cytokines (IL-1β and TNF-α) in the normal or pathological development of fetuses. Despite these findings, additional studies examining the risks of prenatal O_3 exposure are needed to establish the mechanisms by which O_3 affects pregnant mothers and their offspring.

Epilepsy and O_3

The physiopathology of epilepsy mainly describes the imbalance between excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission, which affects normal neuronal activity. This imbalance is caused by genetic and metabolic etiologies (Brailowsky et al., 1989; McNamara, 1994; Tapia and Massieu, 1997) or by exogenous factors that induce the incidence of seizures such as head trauma, inflammatory and infectious processes, or toxic agents, among which O_3 is included (Escalante-Membrillo and Paz, 1997; Neganova et al., 2011; Yuan, 2012).

The olfactory bulb establishes extensive connections with limbic areas such as the amygdala and hippocampus (Martin et al., 2007), which are structures involved in epilepsy (Karasu et al., 2008). O_3 exposure facilitates the development of tonic-clonic seizures induced by amygdala kindling in rats (Escalante-Membrillo and Paz, 1997). Kindling is an experimental model of epilepsy that is induced by the application of a repetitive low-intensity stimulus to the amygdala. The stimulus is applied to other structures such as the olfactory bulb, and a smaller number of stimuli can trigger epileptic seizures (Goddard et al., 1969). The mechanism underlying how O_3 exposure induces high susceptibility in the development of generalized seizures remains unclear. A recent explanation proposes that O_3 can react with proteins and nucleic acids by modifying the activity of some enzymes such as glutamine synthetase (GS) (Berlett et al., 1996). This enzyme regulates

the synthesis of glutamine from glutamate as well as the pharmacological inhibition of decreased glutamine levels and increased glutamate levels in the rat hippocampus (Eid et al., 2008). Previous reports have demonstrated the inhibition of the GS activity by FR generation (Oliver et al., 1990). Although there are have been no reports on the effects of O_3 exposure on glutamate levels in the hippocampus or olfactory bulb, other studies have shown an increase in glutamate and reduction in GABA levels in the striatum of rats exposed to 1.0 ppm of O_3 (Rivas-Arancibia et al., 2003). This most likely explains the inhibition of the GS induced by the generation of FR through O_3 exposure (Kennedy et al., 1992).

Thus, we emphasize the need for more detailed studies aimed at elucidating the mechanisms by which O_3 facilitates the development of tonic-clonic seizures (Escalante-Membrillo and Paz, 1997). Studies on the effects of O_3 exposure on the olfactory bulb will explain some of the mechanisms related to epilepsies that have olfactory auras, which culminate in seizures (Chen et al., 2003).

Meanwhile, evidence suggests that inflammation plays an important role in epileptic activity. Convulsive seizures in different models increased the IL-1β and TNF-α levels in the brain (Plata-Salaman et al., 2000; Jankowsky and Patterson, 2001), and a similar increase in the expression of IL-1β in epileptic patients has been observed (Lehtimaki et al., 2007). IL-1β and TNF-α share several signaling mechanisms related to the modulation of seizure activity, thus contributing to neural excitability and neurodegeneration (Wang and Shuaib, 2002; Andrzejczak, 2011). The mechanisms proposed involve the IL-1β in epileptic activity and describe an increase in NO formation and in the susceptibility of seizures. Neuronal excitability is also increased by the inhibition of GABAa receptors and by the increase in the production of *N*-methyl-D-aspartate (NMDA) receptors (Wang et al., 2000; Viviani et al., 2003; Zhu et al., 2006). The influx of calcium in the pyramidal cells of the hippocampus upon exposure to NMDA alters the expression of the NR2B subunit. This subunit regulates calcium permeability and induces hyperexcitability (Viviani et al., 2003). TNF-α may also increase AMPA receptor density in the neuronal membrane, which results in calcium entry (Stellwagen et al., 2005). The mRNA levels of TNF-α increased in the parietal, prefrontal, and piriform cortices as well as in the amygdala and the hippocampus of kindled rats (Plata-Salaman et al., 2000). Moreover, low concentrations of TNF *in vitro* induced convulsive activity (Balosso et al., 2005). Several studies have shown that seizures promote cytokine production, which shares specific mechanisms in the pathogenesis of epilepsy and may explain the increased levels of IL-1 and TNF secondary to exposure to 1 ppm of O_3 in rats.

O_3-induced oxidative damage and alterations in the brain plasticity

Oxidative stress can induce neurodegeneration. Neurodegenerative disorders are associated principally with age and are described as inherited or sporadic forms. Recently, environmental pollution factors such as O_3 have been evaluated as risks for neurodegenerative disorders. Neurodegeneration is a pathological phenomenon that involves the activation of oxidative stress, specific damage of tissue, and cellular death. In the initial mechanism of the neurodegeneration, ROS and RNS participate in the enzymatic inhibition of the proteins that regulate the amino acid excitatory levels and the upper activation of the excitatory receptors as NMDA receptors. Exposure to low doses of O_3 over a long period causes progressive neurodegeneration (Pereyra-Munoz et al., 2006; Rivas-Arancibia et al., 2010). O_3 or ROS derived from O_3 exposure can directly react with amino acids found in proteins and nucleic acids, thus modifying the activity of enzymes (Mehlman and Borek, 1987) that participate in the regulation of amino acids with excitatory activity, such as glutamate and aspartate (Berlett et al., 1996; Rivas-Arancibia et al., 2003). These results propose the exposure of laboratory animals to O_3 as a model for the study of neurodegeneration mainly because of the generation of oxidative stress (Rivas-Arancibia et al., 2003). Alterations in olfactory sensitivity in neurodegenerative diseases and exposure to environmental pollutants, particularly O_3, are related to the structural changes in the granulosa cells in the olfactory bulb, which are considered associative neurons in this brain region (Arnold et al., 1998; Colin-Barenque et al., 2005). Among the early alterations observed in some neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and aging, changes in olfactory sensitivity, such as hyposmia and anosmia, have been described (Hoffman et al., 1998; Wszolek and Markopoulou, 1998; Kovacs et al., 2001). In addition, alterations in regions such as the hippocampus, striatum, and cortex result in alterations of the brain plasticity, such as learning and memory functions, which are regulated by the hippocampus, and in motor behavior involving the striatum and cortex.

Several experimental studies have reported that rats exposed to O_3 have difficulty in performing motor activities and demonstrate a reduced performance in operant conditioning tasks (Tepper and Weiss, 1986; Rivas-Manzano

and Paz, 1999). These studies have shown increases in lipid oxidation products in regions such as the hippocampus and striatum after O_3 exposure. The oxidation of lipids and proteins cause a loss in secondary and tertiary dendritic spines, which is related to a decrease in neuronal plasticity (Avila-Costa et al., 2001; Colin-Barenque et al., 2005). The FR generated by O_3 exposure or by neuroinflammatory mechanisms can attack the nerve terminals, which results in the deterioration of the synapse (Van der Vliet et al., 1995). Rivas-Arancibia et al. (2003) attributed the decrease in motor behavior to changes in the concentrations of NT, such as glutamate, GABA, and 5-HT in the striatum, which is an important brain structure that controls fine movements.

Mechanisms of defense against O_3 exposure

The oxidation products generated by O_3 exposure damage the CNS. Several features make the nervous system uniquely susceptible to these oxidative products (Rusyniak et al., 2005). Nerve cells and neurons, with their long dendrites and axons, have a large surface area for absorption and attack by chemicals (Nelson, 1994). With a dry weight composed of 50% lipids, the brain and nervous tissue are particularly vulnerable to oxidative molecules (Mustafa, 1990; Pryor et al., 1995b). Once injured, neurons and nerve tissue have a limited capacity to regenerate, placing the emphasis for treatment on prevention. The mechanisms of defense against oxidative stress generated by O_3 include antioxidant molecules (Rahman et al., 1991; Bargagli et al., 2009). Antioxidants are biological substances that are able to compete for oxidizable substrates and inhibit oxidation, and antioxidant systems can be divided into enzymatic and nonenzymatic. The former not only includes glutathione, SOD, catalases, and peroxidases but also peroxiredoxin, thioredoxin, glutaredoxin, and hemoxygenases (H-MOX) (Figure 3); the latter mainly includes vitamins (α-tocopherol and ascorbic acid), β-carotene, and uric acid.

SOD is primarily a cytoplasmic defense mechanism and catalyzes the reaction of the superoxide radical ($O_2^{\bullet}$)

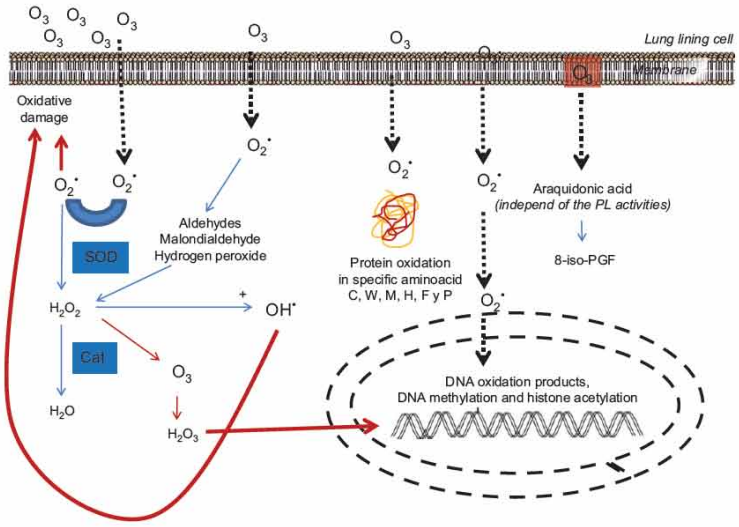

Figure 3 Antioxidant response to O_3 exposure.
O_3 exposure induces an increase in the accumulation of ROS and RNS. The defense mechanisms against oxidative stress generated by O_3 include H-MOX, SOD, and catalase. H-MOX is the first line of defense against oxidants present in the lungs. SOD is primarily a cytoplasmic defense mechanism that catalyzes the reaction of the superoxide radical ($O_2^{\bullet}$) into H_2O_2. The detoxification of ROS is completed by catalase activity, an enzyme that reacts with H_2O_2 and produces H_2O_2. When the balance between the generation of FR and antioxidant systems is broken, an oxidative stress condition develops in which damage to biomolecules occurs. O_3 exposure produces membrane lipid oxidation, which results in reactive aldehyde, oxidation of specific protein amino acids, damage to DNA, and the activation of pro-inflammatory molecules.

into hydrogen peroxide (H_2O_2). SOD has three isoforms: SOD1 (SOD-Cu/Zn dependent) is mainly expressed in the cytoplasm of the epithelial cells, fibroblasts, and alveolar macrophages; SOD2 (SOD-Mn dependent) is mainly mitochondrial; and the third isoform, ec-SOD, is a slightly hydrophobic glycoprotein that binds to cell surfaces and matrix components. In normal lung tissue, ec-SOD is expressed in alveolar macrophages, bronchial epithelium, vascular endothelium, the extracellular matrix, and epithelial cells. Numerous reports have described an increase in SOD2 activity and its expression in models of lung damage induced by chronic O_3 exposure (Weller et al., 1997). Moreover, increases in SOD1 levels after a short O_3 exposure have also been reported (Rahman et al., 1991; Weller et al., 1997).

The detoxification of ROS is mediated by the activation of catalase, a 240-kDa protein, which is mainly expressed in macrophages, pneumocytes, and lung fibroblasts. This enzyme reacts with H_2O_2 and produces H_2O.

H-MOX is the first line of defense against oxidants present in the lungs. *H-MOX-1* is an inducible gene that is present in many tissues, including the lungs, and is self-regulated by increased oxidants (Islam et al., 2008). Reports of exposure to contaminants, particularly O_3, show a genetic susceptibility to the expression of this enzyme after exposure (Islam et al., 2008). Several studies have demonstrated that the expression of a polymorphism containing a series of repeats (GT)n in tandem in the region 5′ of the *HMO$_x$* gene corresponds to a determinant of asthma in people exposed to O_3 (Islam et al., 2008).

The nonenzymatic antioxidant types include vitamins, proteins, and amino acids, which are less reactive but occurs in greater concentration, in contrast to the enzymatic types, which have a high reactivity with the ROS but are in lower concentrations. Vitamin supplements have been reported to reduce the magnitude of symptoms in subjects exposed to oxidant air pollution. However, Mudway et al. (2006) showed that supplementation with vitamins C and E cannot reduce lung function decrements, airway inflammation, and epithelial injury in subjects O_3 exposure.

Conclusion

Being responsible for our thoughts and actions, the nervous system defines us as individuals more than any other organ system in the body. Several features make the nervous system uniquely susceptible to environmental pollution. In industrialized cities, the consumption of different products that are involved in the generation of O_3 are derived from industrial activities as well as from the natural generation of this contaminant, which creates a public health problem. Although data on environmental pollution indicate a decrease in the generation and exposure to adverse health products, it is estimated that air pollution is the eighth highest mortality risk factor, accounting for 2.5% of all deaths in developed countries.

The authorities who control air pollution levels in each country and the WHO have established the limits of O_3 exposure that are considered safe for health; however, a large number of hospital admissions as a result of exposure to environmental pollutants, including O_3, are still being reported. Although the mechanisms by which O_3 exposure induces alterations in the CNS are not fully understood, the most recent reports involve the activation of pro-inflammatory molecules, such as IL-1β and TNF-α. An increase in these molecules has been demonstrated in animals exposed to O_3 at both systemic and CNS levels. Increases in the concentrations of these molecules may also induce alterations reported at the BBB (Teeling and Perry, 2009) in animals exposed to O_3 and may induce the symptoms caused by environmental O_3 exposure, which include irritation of the eyes, nose, throat, respiratory airways, and skin; headaches and fatigue (WHO, 1983, 2005); and alterations in the sleep-wake cycle (Kapsimalis et al., 2008). Moreover, increases in IL-1β and TNF-α, which induce oxidative stress in the CNS, may contribute to an exacerbation of the damage and the generation of FR in the brain parenchyma, which leads to morphological, biochemical, and molecular alterations that together cause the previously described alterations (Teeling and Perry, 2009). Importantly, much of the evidence regarding CNS alterations caused by O_3 is generated from animal models exposed to doses that exceed the permissible limits in humans. Hatch et al. (1994) demonstrated that rodents require much higher doses compared with the exposure doses reported in humans in which neurological disorders due to O_3 exposure were observed (Hatch et al., 1994). These interspecies differences are because, during O_3 exposure, the rats remove a smaller fraction of the inhaled amount of O_3 (40–47%) (Wiester et al., 1987, 1988) than humans do (75% with large inter-individual variations) (Wiester et al., 1996a). Hence, the toxicity of O_3 observed for a given concentration in rodents strongly underestimates the effect observed for the same dose in human.

Because of the publication of the second edition of the WHO air quality guidelines for Europe (WHO, 2000), which sets the guideline value for O_3 levels at 120 μg/m³ for an 8-h daily average, some new information on the health effects

of O_3 has been obtained from either chamber studies or field studies. However, significant additions to the health effects evidence base come from epidemiological time-series studies. Collectively, these studies have revealed small, but convincing, positive associations between daily mortality and O_3 levels, which are independent of the effects of PM. Similar associations have been observed in both North America and Europe. Government initiatives in several countries have failed to control pollutant emissions that generate O_3. Thus far, it has been impossible to reduce the environmental pollution generated by O_3 to levels below those permitted, which causes hazards to public health. Moreover, the generation of O_3 in closed occupational spaces makes it more difficult to eradicate this health problem. Little attention has been placed on O_3 generated within the office environment. It is thought to cause the building-related symptoms (BRS), more commonly known as sick building syndrome, and is characterized by a range of symptoms in office workers, including irritation of the eyes, nose, throat, respiratory tract, and skin; headaches; and fatigue (WHO, 1983). Although these symptoms have not been clearly linked to a specific environmental exposure, a recent analysis by the US EPA Building Assessment Survey and Evaluation showed a correlation between increases in the concentration of O_3 and increased BRS in office buildings (Apte et al., 2008; Buchanan et al., 2008). In recent years, there has also been an increase in the mortality rate associated with O_3, which has resulted in a restatement of the algorithms used to predict mortality and morbidity according to the National Morbidity, Mortality, and Air Pollutions Study (Bell et al., 2006; Chen et al., 2012). Taken together, it is necessary to continue studies on the effects of O_3 exposure particularly in the CNS.

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Chapter 13

SYSTEMIC INFLAMMATION AND NEURONAL DYSFUNCTION PRODUCED BY OZONE EXPOSURE

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ABSTRACT

Ozone (O_3), the major component of air pollution has a significant impact on public health. Besides the described effects on inflammation of the airways, there are many evidences indicating that exposure to O_3 affects the central nervous system (CNS). The biological effects of O_3 are attributed to their ability to cause oxidation and peroxidation of biomolecules directly and/or via free radical reactions, which in turn generates pathological damage to the respiratory system and extra pulmonary effects. The initial concentration and the maximum doses of O_3 in tissues occur in the nose and the surfactant barrier although other effects are localized in the terminal bronchioles and alveolar ducts where the injured membrane generate aldehydes such malondialdehyde, 9 hidroxy-nonenal and other products of reaction of O_3 with unsaturated lipids of membrane and induce the expression of factors such as cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS) which are secreted into the blood. The augmented production of pro-inflammatory products released from injured membranes has been evidenced mainly by the increase of lipid ozonation products (LOP), arachidonic acid (AA), leukotrienes, tromboxanes, TNF-α, IL-1β the Nrf2 and NF-κB transcription factors. All these factors can induce the loss of cells and inflammatory processes in the lung, heart and liver. The loss cells involve the activation and proliferation of neutrophils and macrophages that propagate the effect to other organs including the tissue nearest to the blood-brain barrier (BBB). Furthermore, the activation of inflammatory cells near to BBB produce damage into the BBB and increase pro-inflammatory molecules transported across to the BBB into the brain, producing

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oxidative stress and neuroinflammation. These processes can induce several brain alterations including neurodegeneration. In this review we described the signaling pathways activated as consequence of the environmental exposition to O_3 and their effects on nervous system which produce changes in learning, memory and altered motor activity.

Keywords: Cytokines, ozone, neuroinflammation

EFFECTS OF O_3 EXPOSURE

Ozone (O_3), the major component of photochemical smog, is a highly reactive gas in air pollution that can oxidize many biomolecules in the biological systems. The initial concentration and the maximum dose in tissues occur in the nose and the surfactant barrier. The rate of absorption in human nose is around 40–65% of the O_3 inhaled during quiet breathing [1,2], however only a very small fraction (4-6 %) of the total dose of O_3 can react with cell membranes [3,4] and begins a cascade reaction [5] involving the formation of reactive oxygen species (ROS) [6], accumulation of oxide derivates [7] and inflammatory processes in epithelial cell [8]. Also the transmigration of macrophages, neutrophils and other immune cells occur in subjects exposed to this gas. It has been demonstrated the increase in neutrophils in the noses of subjects after a 4 h controlled exposure to 0.5 ppm O_3 [9]. Also, atrophy of the nasal cilia and hyperplasia of the basal cells were reported in residents living in polluted areas of Mexico City [10].

Respiratory functional tests performed in voluntary patients breathing 0.25 ppm O_3 show severe respiratory symptoms including shortness of breath, irritation of the airways, coughing and chest tightness [10]. At the systemic level, inhalation of O_3 is associated with alterations in the circulatory system as increased heart rate, headaches also enhances the admission to the hospital emergency department for respiratory complications of some diseases such as asthma [11,12,13]. The most common systemic effects of exposure to O_3 has been described in lungs, heart liver and brain [14,15]. The acute effects of O_3 exposure on health are based on controlled exposure studies in which it was determined that a single exposure to O_3 causes changes in respiratory function, these changes are expressed as the increased resistance airways, decreased forced expiratory volume and forced vital capacity determined during one second [16,17,18,19]. In addition, the changes produced by O_3 inhalation include augmented airway reactivity, permeability to macromolecules and infiltration of neutrophils into the area of contact with the O_3 also increments on secretion of mucus in the airways [20,21]. O_3 exposed animals showed the chronic presence of both inflammatory cells and their secretor products, these processes may contribute to the observed hyperplasic and metaplasic processes in the airways [22].

Though the lung is the main focus of damage after O_3 exposure, several studies in humans and animals show that in vivo exposure to O_3 produce injury in extra-pulmonary tissues. Exposition of cells to O_3 produce the presence of factors that increase DNA synthesis in the serum of lung cells in culture [8]. It has been demonstrated that the heart and brain are damaged by a concentration (0.25 ppm or 0.7 ppm for 5 days) of O_3 which are present in major urban centers; they may have important implications for chronic illness and degenerative processes in humans. From these concentrations of O_3 exposure it increase on

levels of thiobarbituric acid-reactive material in the right and left cardiac ventricles tissue as well as elevated activity of catalase and glutathione peroxidase in this tissue [15].

In the liver of mice exposed to O_3 it has been reported the down-regulation of genes to cytochrome P450s this down-regulation is most likely due to the products of reaction of O_3 with substrates in the lung [23] or to long-lived signaling molecules produced by inflammatory or epithelial cells in the lung in response to injury [14]. Most of the down-regulated P450 transcripts code for sterol hydroxylases (1A2, 2A4, 2D10, 2D11, 7B1, 8B1, and 17A1) involved in bile acid and steroidal hormone metabolic pathways, in addition to their roles as xenobiotic monooxygenases [14]. Recently, using global gene expression analyses, investigators found that livers of C57BL/6 mice acutely exposed to inhaled O_3 had significant down-regulation of gene families related to lipid, fatty acid, and carbohydrate metabolisms that were consistent with systemic cachexia in response to O_3 exposure [14]. Transcription of several messenger RNAs (mRNAs) encoding enzymes of xenobiotic metabolism was also decreased in livers of these O_3 exposed mice. Since several interferons (IFN)-dependent hepatic genes were down-regulated with O_3 exposure; the investigators suggested that IFN may act as the signaling molecule between the lung and liver [14,23]. Besides, mice exposed to O_3 have prolonged pentobarbital sleeping time [24] and impairment of hepatic drug metabolism [25].

Despite the evidence, mechanisms by which O_3 produces its effects in organs distant to the area of exposition are not fully understood. These findings led to the speculation that the toxic effects of O_3 are mostly due to the reaction products generated by their contact with the epithelial lining fluid of the lung, where O_3 reacts directly with unsaturated fatty acids [7,26,27,28], causing severe damage to the lipid membrane of the lung [29].

Biomolecules and Ozone Interaction

The biological damage produced by O_3 involves the activation of several mechanisms. The most described mechanism of O_3 reaction is the activated by oxidative destruction of biomolecules either by a direct reaction or through the formation of free radicals (FR), reactive oxygen species (ROS), reactive nitrogen species (RNS) and other reactive intermediates. (Table 1).

Several evidence over the past years showed that lung injury following ozone exposure is due, not only to direct effects of the chemical, but also indirectly to the actions of inflammatory mediators released by infiltrating macrophages [30]. The first reports of lung damage produced by the ozone exposition, indicated that the accumulation of inflammatory cells such as macrophages and leukocytes (PMNs) [9,31], were found near the terminal bronchia, the respiratory bronchioles, the alveolar ducts and proximal alveoli [32].

In lungs, the acute O_3 exposition increases ROS production [33], initially these exposition produce an injury phase involving damage or loss cell associated with depression of enzymatic activity and other metabolic parameters [34]. A deficiency in the assemblage of enzymes that counteract the ROS production can generate atrophy in the ciliated and type I cells from alveolar region [19,32]. Other effects produced are localized at the junction of terminal bronchioles and alveolar ducts, they include an evident loss of cells and accumulation of inflammatory cells [32,35] which induced inflammatory processes in the

lung. The O_3 can cause damage beyond the production of ROS and lipid ozonation products (LOPs) forming aldehydes as the 2-aldehyde-hidroxyhidroperoxide, peroxides and hydroperoxides such as 9-carbon hidroxyhidroperoxide (HHP-C9) that bind to a glycerol form carbon aldehyde 9 (Ald-C9; nonanal) which is released into the extracellular milieu [8,17,36]. In vitro studies have shown that incubation of airways epithelial cells with the aldehyde 9-nonanal increases the activity of phospholipase C (PLC) [37]. O_3 also activates hydrolitic enzymes as phospholipase A2 (PLA2) and PLC to generate arachidonic acid which by the action of cyclooxygenases and lipoxygenases produce prostaglandins and leukotrienes, but also ROS and lipid hydroperoxides [38]. The combination of O_3, nitrogen oxides (NOx), carbon monoxide (CO), lipopolisacharides (LPS) and particles matter (PM) which occurs in photochemical smog produce several effects which may be additive or synergistic. A synergistic lung injury occurs possibly due to a formation of more powerful radicals and chemical intermediates [32,39].

Table 1. Products generates by ozone interaction with cell membranes

	Products generates	Biological effects	Species	References
Formation of Free Radicals and reactive intermediates				
LOP	*Aldehydes hexanal, nonanal, Malonaldehyde*	Chemotactic for polymorphonuclear leukocytes, produce alteration of alveolar macrophages function. Suppress T-lymphocyte mutagenesis, activate eicosanoid metabolism in airway epithelial cells and produce products of the Criegee ozonation process including aldehydes.	Human	[36]
	Protein glutathione mixed disulfide PSSG and PSSP	Modify the status redox, changes in membrane of thiol groups to correlate with alterations in membrane transport functions, permeability, and adenylate cyclase activity	Erythrocyte Ghosts and intact red cells	[4,122]
RNS	*Perxyntrite, NO and iNOS*	Increase of expression induce NO synthesis, NO can react with superoxide anion forming peroxynitrite, a potent oxidant known to nitrosylate tyrosine residues in proteins Increased expression of NOS2 protein and mRNA by alveolar macrophages and increased production of nitric oxide as well as peroxynitrite induced damage.	Mice	[123,124,125]
ROS	*Hydroxyl*	Is a highly reactive radical that cause damage to cells, react directly with DNA, lipids and proteins	Humans, rats, mice, cell cultures	[48,26]
	Hydrogen peroxide	Reactive radical that cause damage to cells, react directly with DNA, lipids and proteins	Mice, rats	[7,126]

	Products generates	Biological effects	Species	References
Loss of functional groups and activities of biomolecules				
	Surfactants Protein A	Induction of the production of tumor necrosis factor TNF-α and interleukin 8 (I L-8)	THP-1cells	[64]
	C fibers	Decrease the response and play an important role in the production of phagocytes (macrophages)	Rats	[43,46, 47]
Inflammatory markers				
	Prostaglandins (PGs)	Biomarker for lipid per-oxidation	Human	[127]
	IL-1α, IL-6, TNF-α, IL-8	Pro-inflammatory cytokines that induce inflammation	Human, rats, mice, cell cultures	[45,44]
	COX-2	Enzyme that increased levels of leucotrienes, tromboxanes and prostaglandins.		[73]
	Neutrophils	Induction of airway inflammation and contributing factor to acute exacerbations of asthma and chronic bronchitis	Human lung	[16]
Induction of transcriptional factors				
	Vascular endothelial growth factor (VEGF)	Promotes cellular recovery following brain injury	Rats	[128]
	Endotelin 1 and 3	Since alteration with NO homeostasis may be implicated in the pathogenesis of cerebrovascular and neurodegenerative disease.	Rats	[129]
	Nuclear factor kappa B (NFkB)	Regulates multiple genes, that increase the synthesis of a potent oxidant and intracellular vasodilator, the inducible nitric oxide synthase (NOS2), that produce nitric oxide (NO) and the inducible form of cyclooxigenase (COX-2).	Rats, mice,	[67,68, 124, 130]
	Erythroid-related factor 2 (Nrf2)	Participate in responses to oxidative stress, Nrf2 protect against the toxicity produced by exposure to electrophilic chemicals and oxidants. Regulate the expression of cytoprotective genes (superoxide dismutase, catalase and the glutathione peroxidase), involved in the detoxification of ROS produced from air pollution.	Rats, mice,	[60, 61, 62]

The interaction of O3 with cellular membranes produce several reaction products that guide the generation of LOPs, RNS, ROS these products induce chemotactic signals for immune cells and activate the response of PMNs, alveolar macrophages and inflammatory markers. The activated macrophages produce NO and cytokines that increases the levels of transcription factors which promote the increase of inflammatory factors and the increase damage in cells.

THE IMMUNE SYSTEM EFFECTS OF O_3 EXPOSURE

The immune response to O_3 exposure is mediated by a number of soluble factors and cells grouped according to their ability to regulate both innate and adaptive responses. Innate immunity depends mainly on mechanisms effectors of phagocytosis that include humoral factors, complement factors and lysozymes, which can respond to different infectious agents. In contrast, acquired immunity is specific to a particular pathogen, and is characterized by its memory capacity, which improves the response to repeated encounters with the pathogen. Then, O_3 exposure increases the amount of macrophages and neutrophils in the airways [40,41,42], the amount of platelet activating factor (PAF) [43], levels of tumor necrosis factor (TNF) [44], the production of interferon gamma (IF-γ) [45], and decreases the response of C-fibers [46,47,48], playing an important role in the production of phagocyte cells such as macrophages [41]. O_3 stimulates the production of leukocytes, which can induce allergic reactions [42]. It also stimulates the production of interleukin-2 (IL-2) [49] necessary for the production of T lymphocytes; O_3 also stimulates production of antioxidant enzymes [50]. Exposure of epithelial cells generates O_3 dose-dependent increases in the phospholipase activity (PLA2, PLC and PLD) [51]. The phospholipase activity is dependent of G proteins, suggesting that epithelial cells can respond to O_3 through signaling pathways that involve activation of G protein receptors [8].The activation of this pathway increases the release of other mediators, among which the eicosanoids, the prostaglandin E2 (PGE2), the factor PAF [43,52,53], IL-6, IL-8, the reactive oxygen species [8,54] and other cytokines involved in the inflammatory response [31,55] have been found.

The O_3 inhalation also activates the receptor for interleukin-1 (IL-1R) [56] and the Toll-like receptor type 4 (TLR-4) [48,57] activated by endotoxins [58], using similar mechanisms of signaling. Activation of these receptor complexes leads to the activation of transcription factors such as the ubiquitous and pleiotropic nuclear factor-kappa B (NF-κB) [56,59] and the erythroid-related factor 2 (Nrf2) involved in responses of cells to oxidative stress and regulation of expression of cytoprotective genes (superoxide dismutase, catalase and glutathione peroxidase), and detoxification of ROS produced from air pollution [60,61]. The O_3 inhalation increases the activity of Nrf2 and cause the dissociation of the transcription factor Nrf2 from the protein Keap1 [62,63], inducing that Nrf2 can move to the nucleus and activate the gene transcription that limit ROS toxicity, inflammation and immune responses [60,61,62].

THE INFLAMMATION: A CONSEQUENCE OF IMMUNE SYSTEM ACTIVATION PRODUCED BY EXPOSURE TO O_3.

The consequences of exposure to O_3 involve general mechanisms of the immune system activation that produces inflammation. The inflammatory response is generated by the activation of immune system to inactivate or destroy invading organisms, remove irritants and repair damaged tissue. The inflammatory response consists of a series of immunological reactions. The primary processes in inflammation include the increased vascular permeability and the release of lipid-derived, such as eicosanoids or PAF, large peptides, such as IL-1,

small peptides such as bradykinin, and amines like histamine or 5-hydroxytryptamine from damaged tissues and migrating cells [16,30]. The second processes involve the increase in the transcription of the factor NF-κB that regulate inflammatory and immune responses and modulates apoptosis in response to cellular stress [64,65,66]. The NF-κB regulates multiple genes [67,68], that increase the synthesis of a potent oxidant and intracellular vasodilator, the inducible nitric oxide synthase (iNOS, NOS2) [48,69,70,71,72], that produce nitric oxide (NO) and the inducible form of cyclooxigenase (COX-2) [73], enzyme that increased the levels of leucotrienes and tromboxanes. NO participates significantly in almost all stages of the development of inflammation in particular in regulating the properties for the endothelium and inflammation in the early stages of the transmigration of inflammatory cells at sites of inflammation [74]. The disruption of nNOS gene expression reduce the oxidative natural damage [75], this evidence suggest that the inhibition of NOS activity can be regulated the by oxidative damage produce by O_3 exposure [76]. The NF-κB increases the expression of proteins including cytokines, chemokines, major histocompatibility complex (MHC), receptors required for adhesion and migration of neutrophils [66,73,77,78]. Cytokines such as IL-1 and TNF-β can directly activate the NF-κB pathway; this can result in a loop that contributes to the amplification and persistence of the inflammatory response producing chronic inflammation in local sites.

The exposure to O_3 induce increase in the levels of NF-κB and pro-inflammatory markers such TNF, interleukins IL-1, IL-2, IL-6, IL-8, cytokine-induced neutrophil chemoattractant (CINC-1) and the monocyte chemoattractant protein 1 (MCP-1) in bronchial fluid and alveolar epithelial cultures [49,79]. These observation suggest an important role in the family of proteins NF-κB in mediating the pulmonary effects of O_3 [80] This can be a possible mechanism for the occurrence of systemic effects in mice exposed to O_3, because that all products generated by NF-κB, can travel freely in the bloodstream and cause changes in other organs including the brain-blood barrier (BBB) and the central nervous system (CNS).

Potential Neuroinflamatory Pathways Activated after O_3 Exposure

The systemic increase of LOPs, inflammatory factors ROS and RNS leads to effects that occur within the CNS after acute or chronic exposure to O_3. The penetration of substances into and out of the brain is strictly regulated by the BBB. The restricted permeability of this barrier, formed by brain capillary endothelial cells, is a decisive condition for maintaining the integrity of the brain. Animal studies investigating O_3 exposure showed that astrocytes localized near brain capillaries have enhanced expression of IL-6 and TNFα [81]. In addition, astrocyte exposure to ozone in vitro results in astrocyte death [82]. However, it is unclear how astroglia in the brain are activated; this indicate that astroglia respond to components of air pollution, to the inflammation and oxidative stress produced from other cell types, or to cellular damage [83].

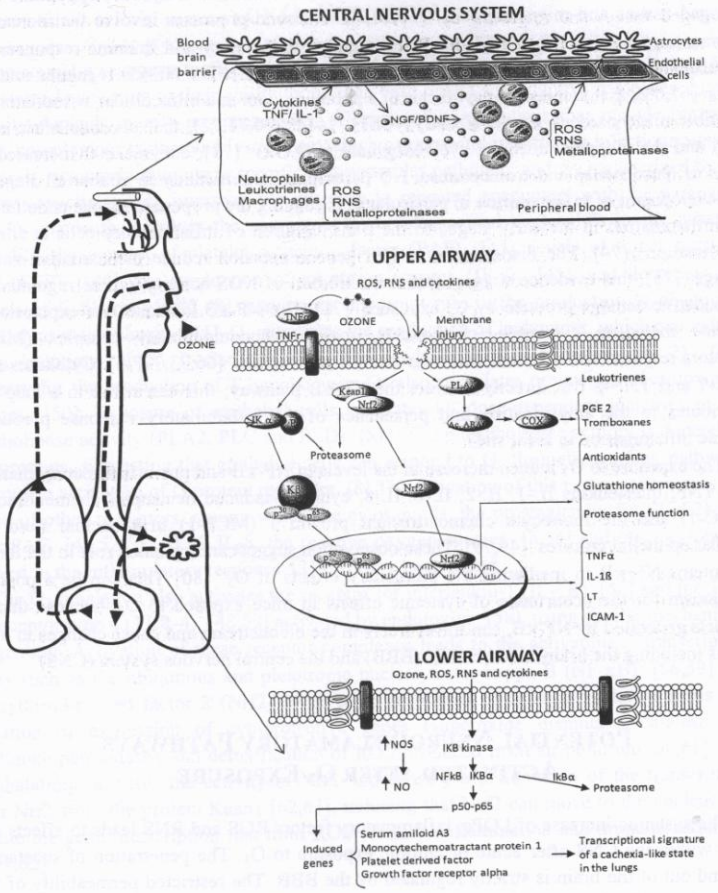

Figure 1. Signaling pathways involved in the damage produced by O_3 exposure and molecular effects in the CNS. The figure shows the mechanisms of damage in the lower airway, upper airways, CNS and the molecular effectors produced by O_3 exposure. The *upper airways* are the first contact target, the O_3 induce the LOP formation followed by ROS, RNS and cytokines, after the injury in cellular membranes; the increase the IKB-kinase that active NF-kB producing the IKBα dissociation. When the p50-p65 from NF-kB translocate into the nucleus, induce the gene transcription of several molecular effectors as amiloid A3, monocyte chemoatractant protein 1, platelet derived factor and grow factor receptor alpha. These molecules can be considerate second messengers that activate the *lower airways* where initiate the TNFα cascade reaction dependent to TNFα receptor activation besides, the membrane injure induce the PLA2 activity and release araquidonate with the production of COX. The pro-inflammatory molecules produced by COX or TNFα pathways induce the inflammatory cellular response to systemic level; this produce injure at BBB as well as exacerbation of the excitotoxic damage and activation of the inflammatory response within the CNS. In *CNS* the inflammatory markers can produce damage in the BBB and the circumventricular organs and cross into the brain inducing the propagation of the inflammatory damage by activated astrocytes.

Breakdown of the BBB and nasal respiratory olfactory epithelium facilitates the access of systemic inflammatory mediators and components of air pollution to the CNS [84]. Given the significant role the BBB plays in regulating normal immune function and inflammatory responses in the CNS, there is a significant interest in factors that may alter normal BBB function, and hence affect the normal immune status in brain [85,86]. Following acute insults to the brain, BBB function may become compromised allowing the entry of immune cells from the circulation [87].

Transmigration of leukocytes following BBB disruption may result in the activation of glial cells in the CNS, the passage of leukocytes to the brain following the red blood cells (RBC) is likely to be increased in subjects exposed to pollutants owing to the disruption of the BBB as described in previous lines, and it is likely related to the production of NO [88]. Both the infiltrating peripheral immune cells and activated glial cells engage in the production of cytokines, promoting inflammation [89].

In normal conditions the cytokines secreted by the immune system in the periphery cannot cross the BBB, however, there is evidence that molecules produced in the acute and chronic exposure to O_3 can share from the circulation to the brain through tree access pathways: a) damaged areas of the BBB [89], b) the choroid plexus [83] and c) circumventricular organs [90]. There is also the active transport of these molecules by endothelial cells transporters. Another way to spread the signal from the periphery to the CNS is based on the cytokines generated de novo in endothelial cells of the BBB, these cells depending on the stimulus and the cytokines involved, produce several soluble factors which activate directly to neurons or indirectly through microglia and astrocytes (Figure 1) [91].

The cytokines regulate the feedback loop between glucocorticoids and the hypothalamic-pituitary adrenal axis (HPA); also regulate several neural circuits and some physiological processes such as thermoregulation, food intake and sleep patterns. The immune system and nervous system are involved at various levels, the main communication between the CNS and the immune system is the HPA [92,93] the major described cytokine that activates the HPA axis is IL-1 however the activation of the axis can be made from other cytokines [94]. This pathway is activated in response to stress; the effects of this activation in the brain are evidenced by the release of corticosteroids in the blood.

These hormones not only mobilize energy reserves it also reduce the number and function of lymphocytes, which generates its immunosuppressive effects [95]. The current consensus is that the pro-inflammatory cytokines, in particular, IL-1β , TNF-α and IL-6, generated in the periphery, go to the CNS, where they induce synthesis of more cytokines and other pro-inflammatory molecules [96,97,98] . Cytokines modulate neuronal circuits involved in various responses such as fever and changes in behavior (anorexia, loss of activity and depression) [99,100].

The inflammatory response in the nervous system is regulated by humoral immunology for IL-10 [101], norepinephrine [102] and alpha-melanocyte-stimulating hormone [103]. The activation of afferent neurons from vague alert the CNS from the presence of peripheral inflammation which can cause the activation of the HPA axis and an anti-inflammatory response to counter the swelling [104].

EFFECTS OF REACTION PRODUCTS GENERATED BY EXPOSURE TO O_3 IN THE CNS

The effects of inhalation of O_3 in the CNS described in controlled human exposure to different concentrations of O_3, include lethargy [105], fatigue and headache [106], memory impairment [107], also in experimental models the effects include, decreased motor activity [76, 108, 109], increased levels of lipid peroxidation [76, 108], changes in levels of neurotransmitters (NT) as the gamma aminobutyric acid [108], glutamate [110], nitric oxide [108], serotonin [111, 112] and norepinephrine [113, 114]. The reports in enzyme synthesis and degradation of NT, the activity of tyrosine hydroxylase [115] is inhibited after exposure to O_3. The changes in NT are correlated with their regulated physiological processes, so, the changes found were, disruption of sleep patterns in cats and rats exposed to different concentrations of O_3 [111, 112, 116]. These effects on sleep are reversed with the administration of indomethacin, a non-steroidal anti-inflammatory [117]. Changes in the concentration of NT can also produce morphological alterations as demonstrated in the cerebellum of rats exposed during pregnancy [118], which are related to changes in norepinephrine concentration of animals prenatally exposed to O_3 [114]. All these findings are generated from exposure to different concentrations of O_3. Also effects related to neurodegeneration after chronic exposure to O_3 were assessed as the presence of the oligomeric forms of amyloid derived diffusible ligand [119], oxidative stress in hippocampus and substantia nigra [110, 120]. Far failed to determine the mechanism by which O_3 produces these changes in the CNS, however, it is proposed that the reaction products generated by exposure to O_3 enter to the CNS through the BBB disruption, active transport and translocation along the olfactory nerve in the olfactory bulbs [83, 89, 121], once in the CNS, altered their homeostasis.

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