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Induction of redox changes, inducible nitric oxide synthase and cyclooxygenase-2 by chronic cadmium exposure in mouse peritoneal macrophages☆

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Abstract

Redox changes and the secretion of inflammatory mediators were investigated in resident peritoneal macrophages of mice chronically exposed to cadmium (Cd, 15 ppm for 2 months) through drinking water. Our results showed that in vivo Cd exposure altered the redox balance in mouse peritoneal macrophages, leading to excessive production of reactive oxygen species (ROS) that overwhelmed the antioxidant defenses. It also led to increased lipid peroxidation and arachidonic acid (AA) release, higher nitric oxide and prostaglandin E_2 (PGE_2) production, and induction of inducible nitric oxide synthase and cyclooxygenase-2 compared with control macrophages. Oxidative stress and inflammation could be important processes operating in the modulation of mouse macrophage physiology induced by chronic Cd exposure.

Keywords: Chronic cadmium exposure; Mouse peritoneal macrophage; Oxidative stress; Inducible nitric oxide synthase; Cyclooxygenase-2

1. Introduction

Oxidative stress, an excessive production of reactive oxygen species (ROS) that overrides the antioxidant systems, can damage cells by lipid peroxidation and alter protein and nucleic acid structure and function (Sevanian and Hochstein, 1985; Spatz, 1992; Jansen et al., 1993). In eukaryotic cells, oxidative stress conditions are induced by a variety of environmental hazards, including ionizing radiation and exposure to xenobiotics and heavy metals such as cadmium (Cd) (Kaul and Forman, 2000; Stohs et al., 2000).

Cadmium is a type D heavy metal of great toxicological significance for the environment and living organisms (Nordberg and Nordberg, 2002). It accumulates in the body with age and has an extremely long biological half-life. Because Cd has become widely employed industrially, for example, in electroplating plants, the dyestuffs industry, and the metallurgical and mining industry, it could pose a major threat to the human environment in the form of

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accumulation in tissues and subsequent development of pathologies (Satarug et al., 2003). A particularly insidious route of exposure to excess Cd is drinking water, especially in third world countries. For example, even when a drinking-water guideline value of 5 μg/l has been set (WHO, 1984), international waters sometimes contain Cd concentrations in excess of 0.2 mg/l (Satarug et al., 2003).

In humans, chronic occupational and non-occupational Cd exposure has been reported to cause severe damage to the nervous, endocrine and immune systems, and to promote both the aging process and cancer (Jarup et al., 1998; Nordberg and Nordberg, 2002; Satarug et al., 2003). Cd exposure has also been shown to produce mutagenesis, teratogenesis, neuro-immuno-endocrine modulation, and carcinogenesis, depending on chemical species, animal, administration route and exposure condition (Zelikoff et al., 1994; Jarup et al., 1998; Waalkes, 2000; Satarug et al., 2003).

Two factors that could play a key role in Cd-induced as well as other types of chemical carcinogenesis are oxidative stress and inflammation (Manca et al., 1994; Klauning et al., 1998; Landolph, 2000). In order to prevent oxidative damage such as that induced by Cd exposure, mammalian cells have developed an elaborate antioxidant defense system. In this system, oxidative stress induces both non-enzymatic antioxidants, such as reduced glutathione (GSH) and metallothioneins (MT) (Ochi et al., 1988), and antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Gupta et al., 1991; Hayes and McLellan, 1999).

In immune and inflammatory responses, macrophages are activated (Auger and Ross, 1992) and secrete increased amounts of cytokines, ROS, reactive nitrogen species such as nitric oxide (NO) (Ding et al., 1988), and eicosanoids such as prostaglandin E_2 (PGE_2) (During and Worlitszch, 1995). These substances all contribute to tissue damage mediated by activated macrophages (Laskin and Pendino, 1995). In addition, the expression of cyclooxygenase type 2 (COX-2) has been characterized in adherent mouse peritoneal macrophages, an expression that is enhanced by stimulation of arachidonic acid (AA) metabolism and auto-amplified by PGE_2 (Tordjman et al., 1995).

Both NO and PGE_2 are inflammatory and immunomodulatory mediators in mammalian physiology (Moncada et al., 1991; Reilly et al., 1998); they also play an important role in chemical carcinogenesis (Landolph, 2000). Several oxidative stressors can induce the co-expression of inducible nitric oxide synthase (iNOS) and COX-2, which synthesize NO and PGE_2, respectively (Feng et al., 1995; Swierkosz et al., 1995; O'Banion, 1999). A number of studies have indicated that iNOS and COX-2 expression pathways are also co-induced in vivo in different models that involve both inflammation and oxidative stress conditions (Vane et al., 1994; Swierkosz et al., 1995).

We have previously reported that in vitro Cd exposure in non-cytotoxic conditions induces iNOS expression (Ramirez et al., 1999) and enhances lipid peroxidation, arachidonic release and GSH release from mouse peritoneal macrophages (Ramirez et al., 2001). In addition, Suzuki et al. (1989) and Romare and Lundholm (1999) have separately reported that in vitro Cd exposure induces COX-2 expression and PGE_2 production in mouse osteoblasts.

In previous studies of the effect of in vivo exposure to cadmium through drinking water, we found an increased level of circulating lipids and lipid peroxides in mouse blood, suggesting an increased lipid turnover or removal (Ramirez and Gimenez, 2002). In the present study, to test our hypothesis that Cd toxicity works through oxidative and inflammatory mechanisms, we have explored the effect of Cd exposure in mice and the molecular mechanism underlying its inflammatory effects in peritoneal macrophages. We have demonstrated that chronic Cd exposure through drinking water induces redox changes in favor of pro-oxidant conditions, and increases synthesis of NO and PGE_2 in mouse peritoneal macrophages.

2. Materials and methods

2.1. Chemicals

Cadmium chloride (as $CdCl_2 \cdot 21/2H_2O$) of 99.5% purity was obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM, phenol red-free), Hank's buffered saline solution (HBSS), and fetal calf serum (FCS) were purchased from GIBCO BRL. The Colorimetric Nitric Oxide Assay Kit, rabbit anti-iNOS mouse macrophage, and protease inhibitor cocktail (Set I)

were purchased from Calbiochem. 3-(4,5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), thiobarbituric acid (TBA), lipopolysaccharide from *Escherichia coli* serotype O55:B5 (LPS), and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All reagents to which macrophages were exposed in the culture were determined to be free from detectable LPS by *Limulus* amebocyte lysate assay (Etoxate, Sigma, MO).

2.2. Animals and chronic cadmium exposure

Male Balb/c mice (40±5-days-old) were maintained under standard laboratory conditions with ad libitum access to food (Cargill, Buenos Aires, Argentina). They were kept in groups of five animals each in a 22–25 °C controlled environment with a 12-h light:12-h darkness cycle. Chronic Cd exposure was induced by administration (ad libitum) in the drinking water of 15 ppm of Cd (as $CdCl_2$) for 2 months. Cadmium in drinking water and food of control mice was not detectable, as assayed by inductively coupled plasma-activated emission spectrometry (ICP-AES) as described previously (Ramirez et al., 1999). After mice were starved for 12 h, they were sacrificed under diethyl ether anesthesia. Blood samples were collected from the heart, coagulated and centrifuged to obtain serum, which was either processed immediately to determine lipid peroxidation, or frozen at −70 °C until used for NO and PGE_2 determinations (see below).

2.3. Isolation and culture of mouse peritoneal macrophages

Resident peritoneal macrophages were prepared in a LPS-free system as described previously (Pabst et al., 1982). After washing the isolated peritoneal cells twice with cold HBSS, they were suspended in DMEM, and 2×10^6 cells were placed in each well of six-well culture plates (Corning Glass Works, Corning, NY, USA). After incubation for 2 h at 37 °C in a 5% CO_2/95% air atmosphere, non-adherent cells were removed by vigorously washing three times with ice-cold HBSS. Peritoneal macrophages (adherent cells) from control and intoxicated mice were further cultured 12 h in DMEM supplemented with 1% FCS, 50 μg/ml gentamicin, and 50 μg/ml fungizone. This incubation permitted the cellular metabolism to adapt to the culture conditions. After incubation, 150 μl of ice-cold 50 mM Tris–HCl, pH 7.8 containing 0.01% Triton X-100 and protease inhibitor cocktail (Calbiochem) was added to the monolayers; cells were scraped, vortexed and centrifuged at $10\,000 \times g$ for 10 min at 4 °C in order to obtain cell homogenates. The protein content in the resulting supernatant (macrophage homogenate) was determined according to Wang and Smith's method (Wang and Smith, 1975) using BSA as a standard.

The Cd content in macrophages after the intoxication period was determined by ICP-AES as previously described (Ramirez et al., 1999). The MTT reduction assay did not show significant cytotoxicity after the incubations described in this research.

2.4. Determination of reactive oxygen species

2.4.1. Superoxide anion production

The production of O_2^- by intact cells was measured as the SOD inhibitable reduction of cytochrome *c* (Pick and Mizel, 1981). Briefly, after the culture stabilization, peritoneal macrophages from control or intoxicated animals (0.5×10^6 cells/well) were covered with 100 μl of HBSS containing 160 μM ferricytochrome *c* during 90 min at 37 °C. After completion of this incubation, the absorbance was measured at 550 nm in a microplate reader (Benchmark, Bio-Rad). The specificity of cytochrome *c* reduction was controlled by inclusion in each sample of a duplicate containing 300 IU/ml SOD.

2.4.2. Hydrogen peroxide production

The functional activation of macrophages was measured according to Pick and Mizel (1981) procedure based on the phenol red assay, which evaluates the activated state of cells by measuring the HRP-catalyzed oxidation of phenol red by H_2O_2 (hydrogen peroxide, the conversion product of O_2^-) in a product, the absorbance of which was recorded at 610 nm. Briefly, control or intoxicated macrophages (0.5×10^6 cells/ml, in 96-well plates) were stabilized for 12 h, then washed with HBSS, covered with 100 μl of phenol red solution (PRS, HBSS containing 0.28 mM phenol red, and 19 IU/ml HRP) and incubated for 60 min at 37 °C. Controls included wells with PRS but no stimulant

and wells with PRS but no cells. After incubation, the monolayer proteins were determined. The absorbance of the resulting colored product was measured on a microplate reader at 610 nm. A standard curve was made, using the same batch of PRS, with H_2O_2 solutions ranging in concentration from 1 to 60 μM.

2.4.3. Determination of hydroxyl free radical production

Macrophage homogenate was used for studying the hydroxyl free radical production, by using dimethyl sulfoxide as a molecular probe, following the methane sulfinic acid (MSA) production as previously described (Babbs and Steiner, 1990).

2.5. Determination of antioxidants

2.5.1. Catalase assay

The CAT (EC 1.11.1.6) activity was measured according to the method of Aebi (1983). The rate of H_2O_2 decomposition was followed by monitoring absorption at 240 nm in 50 mM phosphate buffer, pH 7.0, containing 10 mM H_2O_2 at 25 °C. The activity of CAT is defined as μmol of H_2O_2 consumed per min per mg of homogenate protein. H_2O_2 concentration was calculated using a mM extinction coefficient for H_2O_2 of 0.0394.

2.5.2. Glutathine peroxidase assay

The GPx (EC1.11.1.9) activities in tissue homogenates were determined by an indirect, coupled test procedure (Lawrence and Bork, 1976). Briefly, the GSSG produced during the GPx enzyme reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was monitored as a measurement for the rate of GSSG formation during the GPx reaction. The enzyme reaction was conducted in buffer containing 20 mM potassium phosphate, pH 7.0, 0.6 mM EDTA, 0.15 mM NADPH, 4 IU/ml glutathione reductase, 2 mM GSH, 1 mM sodium azide, and 0.1 mM H_2O_2 at 25 °C, and the rate of decrease in the absorption of NADPH at 340 nm was followed. The GPx activity is defined as nmol NADPH consumed per min per mg cell homogenate protein at 2 mM GSH. Consumption of NADPH was calculated using a mM extinction coefficient for NADPH of 6.22.

2.5.3. Assay for superoxide dismutases (EC 1.15.1.1)

Activities of copper–zinc and manganese SODs were determined by measuring the inhibition of xanthine plus xanthine oxidase-mediated cytochrome *c* reduction at pH 7.8 at 25 °C (Flohé and Otting, 1984). The measurement was performed in the presence of 10 μM KCN to eliminate the activity of tissue cytochrome *c* oxidase. To distinguish the contribution of Cu,Zn-SOD and Mn-SOD to the total SOD activity, the same measurement was also repeated in the presence of 5 mM KCN to inhibit the activity of Cu,Zn-SOD. One unit of SOD activity is defined as the enzyme activity needed to inhibit 50% of the cytochrome *c* reduction.

2.5.4. Glucose-6-phosphate dehydrogenase assay

Glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.2.49) activity was measured by following the coupled NADPH production at 340 nm in a Tris–HCl 0.1 M solution of pH 7.6 at 37 °C (Glock and Mc Lean, 1953).

2.5.5. Thiol antioxidant assay methods

The levels of GSH and its oxidized form (GSSG) contained in macrophages were determined using a kinetic assay in which catalytic amounts of GSH, GSSG and glutathione reductase bring about the continuous reduction of 5,5′-dithiobis(2-nitrobenzoic acid) by NADPH oxidation (Akerboom and Sies, 1981). The MT content in macrophages (2×10^7 cells) was determined by using a silver saturation method according to Scheuhammer and Cherian (1991).

2.6. Determination of lipid peroxidation

The lipid peroxidation level in mouse serum and in the macrophage culture (2×10^6 cells/ml) induced by chronic Cd exposure was correlated with the content of thiobarbituric acid reactive substances (TBARS), principally malondialdehyde (MDA), and measured as previously described by Jentzsch et al. (1996).

2.7. Arachidonic acid labeling and release experiments

Peritoneal macrophages were obtained from control and intoxicated animals and incubated for 12 h in DMEM. They were then labeled by incubating with

0.1 μCi [^{3}H]AA in 1 ml of DMEM for 6 h. After that, monolayers were washed three times with HBSS containing 0.5% BSA and incubated in 1 ml of DMEM containing 10 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, antibiotics, and 0.5% BSA. After 6, 12, 18, 24, 36 and 48 h, the medium was removed, placed in a scintillation cocktail, and its radioactivity counted. The total release of free [^{3}H]AA and its radiolabeled metabolites (radioactivity release) was measured by liquid scintillation spectrometry and expressed as percentage with respect to control cultures.

2.8. Nitric oxide and PGE_2 determination

The levels of NO and PGE_2 in the serum of control and intoxicated animals were determined to compare the systemic effects of Cd exposure with the effect on the macrophages. NO and PGE_2 were determined in serum (hemolysis-free) or supernatant from macrophage cultures (2×10^6 cells/well), using a colorimetric NO assay kit (Calbiochem) for NO and a competitive PGE_2-ELISA assay (Oxford Biomedical Inc.) for PGE_2, and strictly following the manufacturer's instructions.

2.9. Immunoblotting for iNOS and COX-2

Twenty microliters of cell homogenates (2 μg protein/μl) were mixed with equal volumes in *Laemmli* sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 3.5 mM DTT, and 20% glycerol), heated, and loaded into an 8% polyacrylamide gel. Rainbow-colored protein molecular mass markers (14.3–200 kDa) were always loaded into each gel. The separated proteins were transferred to nitrocellulose (BioRad) by using a Blot Transfer System (Bio-Rad Laboratories, Hercules, CA). After being blocked with a 3% gelatin–TBS solution (20 mM Tris, 500 mM NaCl, pH 7.5) the blot was incubated either with a rabbit anti-iNOS (1131–1144) mouse macrophage antiserum (Calbiochem) at 1:2000 dilution, or with an rabbit anti-COX-2 murine (C-terminus) antiserum at 1:1000 dilution (Oxford Biomedicals Inc.) for 1 h, followed by five washes with TTBS (0.1% Tween 20, 100 mM Tris–HCl, pH 7.5 and 150 mM NaCl). Bound antibodies were detected by using an Immuno-Blot Assay kit (Bio-Rad Laboratories, Hercules, CA).

2.10. Statistical analysis

All data presented represent mean values ± S.E.M. The statistical analysis included Student's *t*-test and a one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. The level of significance for all statistical analyses was chosen a priori to be $P < 0.05$.

3. Results and discussion

3.1. Chronic cadmium exposure alters redox balance in mouse peritoneal macrophages

Most mammalian cells are continually exposed to ROS, which may be generated extracellularly or intracellularly by a variety of biochemical, physical or chemical agents including, among others: ionizing radiation, toxic xenobiotics (e.g. Cd and other heavy metals), inflammation, and metabolites of membrane lipid transformation (Sun, 1990; Spatz, 1992; Kaul and Forman, 2000). Although Cd is not a Fenton metal and thus, by itself, is unable to generate ROS, free radical scavengers and antioxidants lessen Cd-induced toxicity, suggesting that the heavy metal elicits an increase in free radical production (Stohs et al., 2000).

Resident peritoneal macrophages from mice that were chronically exposed to Cd contaminated water bio-accumulated 6.6 ± 0.4 ng Cd/10^6 cells, as assessed by ICP-AES. In addition, these cells showed a higher production of superoxide, H_2O_2 and hydroxyl free radical (Table 1). Our results agree with the mechanism proposed by Pouahmad and O'Brien, who suggested that the increased production of ROS in hepatocytes exposed to Cd could be a consequence of a disruption in the mitochondrial electron transport chain and/or uncoupling, independent of the cytosolic ROS generated by redox cycling (Pourahmad and O'Brien, 2000). In addition, ROS are powerful prooxidants that are known to be involved in a variety of physiological and pathological processes such as inflammation, aging and chemical carcinogenesis (Klauning et al., 1998; Hogg, 1998).

Total SOD activity in Cd intoxicated macrophages did not show significant changes, but its Cu,Zn-SOD metalloform showed a significant decrease in its specific activity, while the activity of the Mn-SOD

Table 1
Effect of chronic cadmium exposure on redox balance of mouse peritoneal macrophages[a]

		Control[b]	Intoxicated
(A) Production of ROS			
Superoxide anion (nmol/10^7 cells per 90 min)		3.5 ± 0.4	6.9 ± 0.8 (*)
Hydrogen peroxide (nmol/10^7 cells per 60 min)		8.1 ± 1.2	12.3 ± 1.5 (*)
Hydroxyl free radical (nmol MSA/10^7 cells)		0.02 ± 0.01	0.38 ± 0.05 (*)
(B) Antioxidant enzymes[c]			
CAT (μmol H_2O_2/min per mg protein)		4.8 ± 0.3	4.2 ± 0.5
GPx (nmol/min per mg protein)		352 ± 41	279 ± 21 (*)
SOD (μmol/min per mg protein)	Total SOD	380 ± 32	364 ± 43
	Cu,Zn-SOD	267 ± 25	214 ± 18.3 (*)
	Mn-SOD	4.9 ± 0.5	24.3 ± 3.4 (*)
G-6-PD (nmol NADPH/min per mg protein)		20.3 ± 3.1	42.1 ± 3.9 (*)
(C) Non-enzymatic antioxidants			
GSH (nmol/10^7 cells)		31.2 ± 2.7	38.6 ± 3.1 (*)
MT (ng/10^7 cells)		0.06 ± 0.01	0.50 ± 0.07 (*)

In all these studies the analysis were carried out using 10^7 peritoneal macrophages (ca. 1 mg proteins). Data are expressed as mean values ± S.E.M. from three separate experiments, each in triplicate. (*) Asterisk indicates significant differences respect to control by using the Student's *t*-test ($P < 0.05$).

[a] All assays were carried out as described in Section 2 after stabilization incubation for 12 h in medium.

[b] CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase; Cu,Zn-SOD and Mn-SOD correspond to total-, copper zinc- and Manganese-metalloform of SOD; G-6-PD, glucose-6-phosphate dehydrogenase.

[c] GSH, reduced glutathione; MT, metallothioneins.

metalloform increased with respect to control macrophages. CAT activity did not vary significantly. These observations suggest that the higher levels of superoxide anion and H_2O_2 in macrophages of treated animals stem from the failure of antioxidant responses to be induced by the increased levels of ROS, a failure that is connected with the presence of Cd. A number of reports support the suggestion that Cd affects cells via oxidative mechanisms (Ochi et al., 1988; Skoczynska, 1997; Beyersmann and Hechtenberg, 1997; Stohs et al., 2000). When cellular antioxidant capacity (i.e. antioxidant enzymes and sulfhydryl substances) is exceeded by prooxidant production and cannot be increased in response, the result is oxidative stress (Sun, 1990).

Increased lipid peroxidation, the end product of lipid oxidative stress, could be due to decreased GPx activity in macrophages after Cd intoxication, as has been reported in other models (Hayes and McLellan, 1999). We observed a significant induction of sulfhydryl substances, GSH and MT, in intoxicated macrophages; GSH and MT have been considered important thiol reagents that prevent redox changes and chemical carcinogenesis induced by Cd and other xenobiotics in a number of cell models (Ochi et al., 1988; Bannai et al., 1991). The intracellular redox state, which is mainly controlled by GSH and MT, reflects a balance between cellular levels of sulfhydryls and disulfides, which might be altered by ROS and other ROS derived-oxidants (Hayes and McLellan, 1999).

The amount of GSSG in intoxicated macrophages did not vary from that of control cells (<5% of the total glutathione). This failure of the GSSG level to increase under uncontrolled oxidative stress could be a consequence of the induction of G-6-PD (a secondary antioxidant enzyme) in intoxicated macrophages, as shown in Table 1. G-6-PD plays a role in the reduction of GSSG to GSH to maintain the thiol balance and to protect key enzymes from the damaging effects of ROS (Sun, 1990).

Increased lipid peroxidation, one of the major effects of Cd exposure on lipid components (Skoczynska, 1997), was observed in macrophages

obtained from chronically Cd exposed mice, suggesting that antioxidant defenses were overwhelmed (Table 1). It is possible that the higher lipid peroxidation observed in intoxicated macrophages could result from the incorporation of oxidized lipids circulating in the medium. However, given that the control cells exhibited no such increase, it is much more likely that the pro-oxidant pathways in the macrophages continued to operate during incubation. Regardless of the detailed mechanism, it is clear that chronic Cd exposure alters the redox balance in mouse macrophages.

3.2. Chronic cadmium exposure induces nitric oxide and prostaglandin E_2 production in mouse peritoneal macrophages

Chronic Cd intoxication produced an increase in TBARS (7.5 ± 1.6 vs. 12.3 ± 1.5 nmol MDA/ml, $n = 6$), NO (28.5 ± 3.6 vs. 42.3 ± 2.9 μM, $n = 8$) and PGE_2 (0.7 ± 0.1 vs. 4.2 ± 0.9 ng/ml, $n = 6$) in intoxicated mouse serum compared with those from control animals. These results agree with our major research hypothesis, indicating that oxidative and inflammatory changes could be systemically operating in Cd-induced toxicity in mice.

After stabilization of the culture (i.e. 12 h of incubation in medium alone), macrophages obtained from intoxicated mice showed higher time-dependent NO production and lipid peroxidation than control macrophages (Fig. 1A). This result agrees with the current paradigm to explain Cd toxicity, which holds that the Cd toxicity mechanism operates through oxidative stress, finally leading to lipid peroxidation in several mammalian cells (Manca et al., 1994; Skoczynska, 1997; Stohs et al., 2000).

After the culture was stabilized, more time-dependent AA was released into the culture medium by macrophages from intoxicated animals than by control macrophages, as were more AA metabolites (associated with a release of PGE_2) (Fig. 1B). The role of $cPLA_2$ in oxidative stress conditions has been

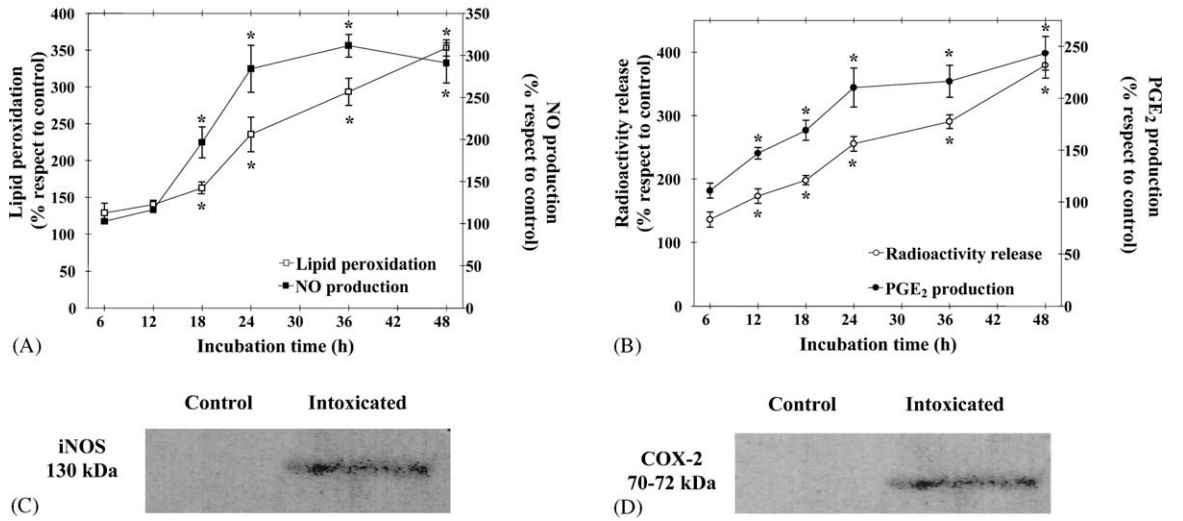

Fig. 1. Chronic cadmium intoxication induces inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in mouse peritoneal macrophages. (A) Time–response curves of lipid peroxidation level vs. NO production; (B) AA and its metabolite release (radioactivity release) vs. relative PGE_2 production; (C) and (D) Western blotting showing iNOS and COX-2 expression, respectively. Each parameter in control vs. experimental mouse peritoneal macrophages was determined as described in Section 2. Each value represents the mean ± S.E.M. from more than three experiments in triplicate. The amounts of NO (ng/ml) and PGE_2 (ng/ml) contained in supernatant of control macrophage culture after 6 h of incubation were 0.2 ± 0.1 and 1.6 ± 0.5, respectively; after 12 h (0.3 ± 0.1; 2.0 ± 0.4); after 18 h (0.5 ± 0.2; 4.5 ± 0.6); after 24 h (0.8 ± 0.1; 5.5 ± 0.3); after 36 h (0.9 ± 0.3; 7.3 ± 0.8) and after 48 h (1.2 ± 0.5; 9.6 ± 0.7). Asterisk indicates significant differences ($P < 0.05$) respect to control cells.

reported previously as responsible for releasing oxidized AA to restore the lipid composition of damaged cellular membranes (Van Kuijk et al., 1987; Muller and Sorrell, 1997). The increased amount of PGE_2 in serum could be a consequence of the increased amount of free and oxidized circulating lipid in Cd-exposed mice (Ramirez and Gimenez, 2002). In addition, in a model of primary mouse osteoblastic cells exposed to Cd, Miyahara and coworkers (Miyahara et al., 2001) found a time-dependent increase in the release of AA, as we found in our in vitro model (Ramirez and Gimenez, 2002); they also found an increase in the expression of $cPLA_2$ and COX-2. A number of reports have indicated that released AA could act as a substrate for prostanoid synthesis or as a signaling molecule in a paracrine or autocrine manner (During and Worlitszch, 1995; Chilton et al., 1996; Balsinde et al., 1998; Gijón and Leslie, 1999).

Many agents that generate oxidative stress induce an adaptive cellular response, represented by an induction in the cellular capacity to challenge any alteration in its physiology (Gupta et al., 1991). We have previously demonstrated that in vitro exposure to Cd under non-cytotoxic conditions induces NO production and iNOS expression, but under cytotoxic conditions it has the opposite effect on both parameters (Ramirez et al., 1999). It is possible that the effect of chronic Cd exposure in mouse macrophages operates through a pathway similar to that of in vitro exposure in non-cytotoxic conditions.

3.3. Chronic cadmium exposure induces iNOS and COX-2 expression

Macrophages obtained from intoxicated animals produced increased amounts of NO (Fig. 1C). Such an effect could be a consequence of higher L-arginine uptake, higher activity of the enzyme, or increased expression of iNOS. Increased L-arginine uptake can be ruled out because the uptake of [^{14}C]-L-arginine did not change significantly with respect to control cells and the amount of L-arginine in the culture medium was the same for control and intoxicated cells (data not shown). Increased enzyme activity in intoxicated cells cannot account for our data. We did not observe any iNOS immunoreactivity in cells obtained from control animals, and if it were only the activity of the enzyme that differed, immunoreactivity should have been observed in both control and intoxicated cells. However, as shown by Western blot analysis, iNOS synthesis was significantly induced in macrophages from intoxicated mice only. This observation together with the increased synthesis of NO suggests that while Cd exposure did not affect the activity of the iNOS synthesized de novo, the transduction pathways maintaining the synthesis of iNOS were not modified and continued to be able to induce additional synthesis in response. Although Harstad and Klaassen found that Cd still produced hepatotoxicity in iNOS knock-out mice exposed to Cd (Harstad and Klaassen, 2002), iNOS induction in macrophages may nevertheless have significant consequences for carcinogenesis.

Macrophages from Cd-exposed mice synthesized more PGE_2 than controls (Fig. 1C). This effect could be due to either a greater release of AA from the membrane by the cPLA or increased synthesis by the key enzyme in the synthesis pathway of this eicosanoid, COX-2. Our previous in vitro studies have already demonstrated that Cd induces a higher release of AA (Ramirez et al., 2001). When we used Western blotting analysis to examine COX-2 expression, we found that the expression pathways induced by in vivo Cd exposure survived after the adaptation incubation. This increased expression could be produced by the redox imbalance observed in intoxicated macrophages with respect to control cells (Table 1).

In agreement with our results, recently Figueiredo-Pereira et al. (2002) used a mouse neuronal cell line to observe a similar relationship between oxidative stress induced by Cd exposure in vitro, as measured by the level of 4-hydroxy-2-nonenal-protein adducts, and the induction of COX-2 and PGE_2 synthesis.

As showed in Fig. 1C and D, the higher NO and PGE_2 synthesis in peritoneal macrophages of Cd-intoxicated mice could be a consequence of iNOS and COX-2 expression, respectively. Various models under oxidative stress conditions have indicated that macrophages and other mammalian cells co-express both COX-2 (Feng et al., 1995; Manca et al., 1994; O'Banion, 1999; Figueiredo-Pereira et al., 2002) and iNOS (Appleton et al., 1996). Increased expression

of these two enzymes would account for the higher PGE_2 and NO generation associated with oxidative stress and inflammation (Swierkosz et al., 1995). In agreement with these results, Suzuki et al. (1989) and later Romare and Lundholm (1999) reported that Cd exposure induces COX-2 expression and PGE_2 synthesis in mouse osteoblasts.

The observed coordinated induction of both enzymes, iNOS and COX-2, might be a cellular effect resulting from Cd induced redox changes. Such an effect could be produced by a transcriptional activator, nuclear factor-κB (NF-κB), that controls the expression of several pro-inflammatory cytokines, as well as the expression of inducible enzymes such as iNOS and COX-2 (Bowie and O'Neill, 2000). Recently, NF-κB has been characterized as a "sensor" of oxidative stress, a sensor that is activated by ROS and inhibited by antioxidants (Li and Karin, 1998).

3.4. Possible significance of redox and inflammatory changes in cadmium induced cellular effects in chronically exposed animals

A number of investigators have demonstrated a coordinated regulation between $cPLA_2$ and COX-2 expression (Balsinde et al., 1998), and iNOS and COX-2 expression under inflammatory conditions (Appleton et al., 1996), such as those found in the chemical carcinogenesis process (Kaul and Forman, 2000). The effect of PGE_2 on iNOS activity and the resulting effect of NO on COX activity have been reviewed previously (Swierkosz et al., 1995).

Cadmium induced chemical carcinogenesis is a medical and social concern, whose mechanism is only partially understood (Waalkes, 2000). Both oxidative stress and inflammation have been observed in a number of models involving environmental hazard toxicity, particularly that induced by heavy metals (Reilly et al., 1998; Manca et al., 1994; Feng et al., 1995; Kaul and Forman, 2000). Cells respond to these adverse conditions by modulation of their antioxidant levels and induction of gene expression (Beyersmann and Hechtenberg, 1997). Our results suggest that oxidative stress induced by a chronic Cd-exposure could be the process responsible for the increased release of pro-inflammatory mediators in resident peritoneal macrophages of intoxicated mice compared with non-exposed animals (Fig. 2).

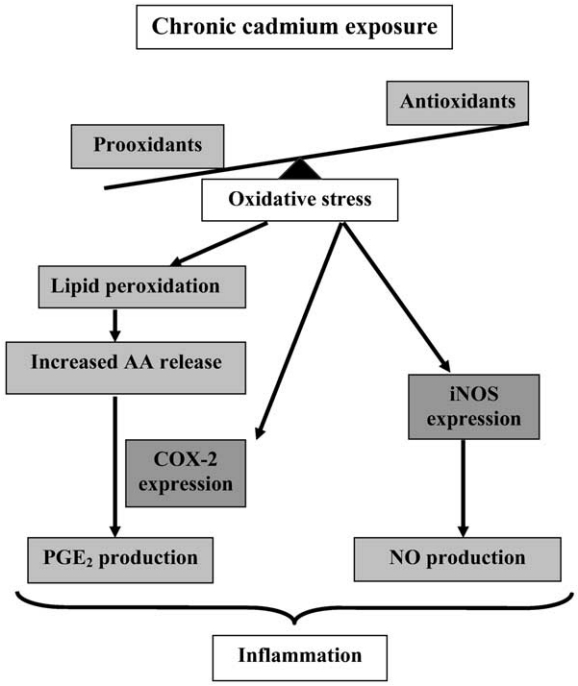

Fig. 2. Oxidative stress and inflammation could be important processes involved in cadmium induced changes in mouse peritoneal macrophages. Cd exposure induces cellular redox changes and then oxidative stress. Oxidative stress produces lipid peroxidation associated to higher AA availability. These changes in the redox environment could activate the nuclear factor-κB (NF-κB) that might induce the expression of the inducible nitric oxide synthase (iNOS) and cyclooxygenase type 2 (COX-2), which produce inflammatory mediators, such as nitric oxide (NO) and PGE_2, respectively.

Oxidative stress and inflammation could be important processes operating in several pathological conditions such as neurodegenerative diseases (Figueiredo-Pereira et al., 2002), as well as in response to a chronic Cd-exposure. These processes could be relevant to the research of chemical carcinogenesis and other effects induced by Cd-exposure. Finally, it seems that depending on the stressor agent or stimulus, redox and inflammatory changes could be associated with a cellular protective response or be toxic action modes. Further studies will be necessary to determine the significance of redox and inflammatory changes on cellular physiology, and to explore

the role of cytokines, adhesion molecules and oxidized mediators on the development of inflammatory changes.

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