Smoking specifically induces metallothionein-2 isoform in human placenta at term

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Abstract

Recently, we reported the presence of higher levels of metallothionein (MT) in placentas of smokers compared to non-smokers. In the present study, we designed experiments to separate and evaluate two isoforms of MT (MT-1 and MT-2) in placentas of smokers and non-smokers. Metallothionein was extracted and separated by ion-exchange high performance liquid chromatography (HPLC), previous saturation with cadmium chloride. Two peaks eluting at 6 and 12.5 min, corresponding to MT-1 and MT-2, respectively, were obtained. Metallothionein present in both peaks was identified by Western blot analysis using a monoclonal antibody directed against MT-1 and MT-2. Each isoform concentration was calculated after measuring its cadmium content by atomic absorption spectrometry with inductively coupled-plasma. In placentas of smokers, MT-2 levels increased by seven-fold compared to non-smokers, whereas MT-1 was not changed. Total placental cadmium and zinc concentrations, determined by atomic absorption spectrometry and neutron activation analysis, respectively, were higher in smokers. Metallothioneins levels were clearly in excess to bind all cadmium ions present in placentas. However, most of placental zinc remains unbound to MTs, although as much as twice zinc ions could be bound to MT in smokers. In conclusion, MT-2 is the main isoform induced by smoking, suggesting that this isoform could be involved in placental cadmium and zinc retention. This fact, which could contribute to reduce the transference of zinc to the fetus, may be associated to detrimental effects on fetal growth and development.

Keywords: Smoking; Placenta; Cadmium; Zinc; Metallothionein isoforms

1. Introduction

Smoking during pregnancy increases the cadmium (Cd^{2+}) body burden, including elevated concentrations of Cd^{2+} in the placenta (Ronco et al., 2005b). Smoking causes a wide range of deleterious effects during fetal growth and development (Ferm, 1971), which may result in low birth weight neonates (Abel, 1980; Ronco et al., 2005b).

Recent studies performed in our laboratory demonstrated that placentas of smoking mothers have increased levels of Cd^{2+}, zinc (Zn^{2+}) and the metal-binding protein metallothionein (MT) (Ronco et al., 2005a,b). These women also delivered neonates with lower birth weights than those delivered by non-smokers. In addition, placental Cd^{2+} levels were statistically and inversely correlated to birth weights (Ronco et al., 2005b). Several studies have reported effects of smoking on fetal growth, but at present, cellular and molecular mechanisms involved in reduced birth weight observed in infants of smokers are not completely understood (Kuhnert et al., 1988a; Roquer et al., 1995). It has been suggested that

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the reduced birth weight found in neonates delivered by smokers may be related to a deficient transfer of Zn^{2+} from placenta to the fetus (Kuhnert et al., 1987b, 1988a). Zinc is an essential micronutrient for fetal growth and for proper immune system function, stressing the importance of Zn^{2+} during pregnancy (Wellinghausen, 2001). When a mother has reduced Zn^{2+} reserves, Zn^{2+} deficiency could occur during pregnancy and nursing due to reduced transfer of this ion through the placenta and breast-feeding. Therefore, maternal Zn^{2+} nutrition is crucial for the infant's Zn^{2+} nutritional status (Dorea, 2002). It is also known that Zn^{2+} supplements reduce childhood morbidity in populations where Zn^{2+} deficiency is common (Hamadani et al., 2002).

Interestingly, it has been shown that smokers, in addition to having elevated placental Cd^{2+} concentrations, also show increased placental Zn^{2+} levels (Kuhnert et al., 1987a; Ronco et al., 2005b). Therefore, the low birth weight of infants born to smoking mothers may be due in part to placental Zn^{2+} accumulation through a mechanism involving placental Cd^{2+} and metal-binding proteins (Torreblanca et al., 1992).

At the cellular level, one of the most important divalent metal (Zn^{2+}, Cd^{2+} and Cu^{2+}) binding proteins is MT. In humans there are 14 MT isoforms with at least 10 functional isoforms (Cherian et al., 2003). Four MT isoforms are the most studied: MT-1 and MT-2 have ubiquitous tissue distribution being abundant in liver, kidney, intestine and pancreas, whereas MT-3 and MT-4 are mainly found in stratified squamous epithelium and brain (Davis and Cousins, 2000). All these four isoforms have been shown to be expressed in rodent placenta (Liang et al., 1996).

Tissue Zn^{2+} accumulation correlates well with MT synthesis (Davis and Cousins, 2000), suggesting that this protein is involved in Zn^{2+} homeostasis by controlling cellular Zn^{2+} uptake, distribution and excretion (Klaassen et al., 1999). Cadmium ions and Zn^{2+}, in addition to manifest high affinity for MT, are potent inducers of MT synthesis (Harford and Sarkar, 1991). Thus, high levels of Cd^{2+} and MT in smoker's placentas (Ronco et al., 2005a) and Cd^{2+}–Zn^{2+}-induced MT synthesis in cultured human trophoblasts have been previously reported (Lehman and Poisner, 1984). In other species, particularly in invertebrates, it has been described that MT-2 is the sole Cd^{2+}-responsive MT and the major isoform involved in Cd^{2+} detoxification (Sturzenbaum et al., 2001, 2004). With all these antecedents, the aim of the present study was to separate and quantify placental MT-1 and MT-2 isoforms to determine whether they are differentially stimulated in smokers and non-smokers.

2. Materials and Methods

2.1. Preparation of placentas

Human placentas ($n = 20$) were obtained upon delivery in the maternity ward at the Sótero del Río Hospital located in southern Santiago. Inclusion criteria included healthy young parturients, with normal pregnancies and without history of alcohol or drugs. All mothers had normal nutritional status evaluated as previously described (Atalah et al., 1997). The ethical committee of our institution approved the research project and the questionnaire, which included medical and dietary history, as well as some data on occupational and environmental sources of metal exposure. The assessment of smoking was based on self-reported individual cigarette consumption and urine cotinine determination immediately before delivery. Parturients were divided in two groups according to their smoking habit: women who never had smoked (non-smokers), and women who smoked during the entire pregnancy (smokers).

Immediately after delivery, the entire placenta was weighed and placed in a sterile plastic bag and frozen at −70 °C. To determine metal elements, half of the partially thawed placenta was thoroughly washed and lyophilized as previously described (Ronco et al., 2005a). Finally, samples were ground and mixed, constituting the stock placental material for metal element determinations. For MT separation and analysis by HPLC, thawed placental tissue was weighted and homogenized.

2.2. Determination of placental levels of zinc and cadmium

In stock placental material, Zn^{2+} was determined by instrumental neutron activation analysis (INAA) at the Laboratories of the Chilean Commission for Nuclear Energy as previously described (Ronco et al., 2005a). The reference sample utilized for Zn^{2+} determinations was pig kidney (trace elements BCR 186, Certified Reference Material, Sigma–Aldrich, MO, USA) with a Zn^{2+} concentration of 128 μg/g.

Cadmium determinations were carried out by atomic absorption spectrometry with a graphite furnace device for solid samples (SS-GFAAS, Carl Zeiss Technology) at the Chilean Commission for Nuclear Energy, as previously described (Ronco et al., 2005a). The reference material used for Cd^{2+} determinations was dorm-2 dogfish muscle (National Institute for Standards and Technology, MD, USA) with a Cd^{2+} concentration of 44.3 ng/g.

2.3. Separation of MT-1 and MT-2 isoforms by HPLC

Placental tissue was weighed (3 g), homogenized in buffer Tris–HCl (10 mM, pH 7.4) and centrifuged at 10,000 × g for 15 min at 4 °C and then at 100,000 × g for 1 h (Nostelbacher et al., 2000). One milliliter aliquot was saturated with 50 μl of 0.11 M $CdCl_2$ (1000 ppm or 5.2 mM $CdCl_2$ final concentration in the placental sample) and heat denatured for 1 min in boiling

water. Subsequently, samples were centrifuged at 10,000 × *g* for 10 min, filtered through an Amicon centrifugal filter unit (30 kDa MW cut-off, Millipore Co., MA, USA) and finally separated and analyzed by high performance liquid chromatography (HPLC) with a UV detector (Merck Hitachi model L-4250). Experimental procedures were performed as briefly described: a 200 μl aliquot of the filtrated solution was injected onto an anion-exchange column EMD DEAE-650 (S) (Merck Fractogel EMD DEAE-650(S), 20–40 μm, 70 mm × 10 mm, with high protein binding capacity). Sample elution was carried out with 20 mM Tris–HCl, pH 7.4 (buffer A) and 200 mM Tris–HCl, pH 7.4 (buffer B) prepared with ultra pure deionized, filtered, double distilled water. Two peaks sequentially corresponding to MT-1 and MT-2 were separated after elution with a continuous linear gradient of buffer B (0–60%) in buffer A, at a flow-rate of 1 ml/min during 25 min. Before any new sample injection, the column was rinsed with 1 M NaCl for 10 min and then with buffer A for 30 min. To quantify MT, different fractions from the HPLC column were collected by means of a fraction collector (Bio Rad model 2110), and Cd^{2+} concentrations of those fractions corresponding to MT-1 and MT-2, were measured by atomic absorption spectrometry with inductively coupled-plasma (AAS-ICP). This last experimental procedure was performed at the Laboratories of the Chilean Commission for Nuclear Energy.

2.4. Determination of MT-1 and MT-2 isoform concentrations

The total Cd^{2+} content of fractions corresponding to MT-1 and MT-2 was utilized to calculate MT-1 and MT-2 concentrations as previously described (Nostelbacher et al., 2000), according to the following equation,

$$\text{nmol MT-}X\text{/g placenta)} = \frac{C \times 7\,(\text{each mol of MT bonds 7 mol of } Cd^{2+}) \times \nu}{M}$$

where X: MT isoform 1 or 2 (MT-1 or MT-2); C: nmol Cd^{2+} per peak (1 or 2); ν: volume of homogenate; M: g of initial placenta (3 g).

Calculation of C (nmol Cd^{2+}/peak)

$$C = \frac{\text{Total } Cd^{2+} \text{ content per peak (ng/ml 1 or 2)} \times 0.2\text{ ml (injection volume)} \times D}{E}$$

where D: dilution of the homogenate saturated with Cd^{2+} (1.05); E: MW of Cd^{2+}: 112.3.

Additionally, to identify the presence of MT in the eluted HPLC peaks 1 and 2, total volumes of these peaks were freeze-dried and subsequently reconstituted in double distilled water and subjected to Western blot analysis.

2.5. Identification of MT in peaks 1 and 2 by Western blot analysis

Western blots were assessed as previously described (Ronco et al., 2005a). Protein concentration was determined by the BioRad protein micro-assay which is based on the method of Bradford, utilizing in this case bovine gamma globulin as the standard protein. Then, 20 μg of protein contained in eluted samples (from peaks corresponding to MT-1 and MT-2) was run in SDS/PAGE electrophoresis (12%; Laemmli, 1970). After 2 h, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and subsequently incubated overnight at 4 °C with a monoclonal anti-MT antibody (anti-MT-1 and anti-MT-2, Dako Corp., USA) diluted 1:1000 in 1% BSA. These membranes were subsequently washed and incubated for 1 h at room temperature with a second anti-mouse antibody conjugated to alkaline phosphatase (AP). Bands were visualized using 5-bromo-4-chloro-3-indolylphosphate disodium salt/*p*-nitroblue tetrazolium chloride (BCIP/NBT; Calbiochem), to detect the product of AP activity.

2.6. Statistics

Statistical analyses were performed using a statistical software package (Statistica for Windows Release 6, Statsoft Inc. 1984–2004, USA). Results obtained from 10 smokers and 10 non-smokers were expressed as median values. The non-parametric Mann–Whitney *U*-test was applied to compare data from both groups, and significance was assumed at $p < 0.05$.

3. Results

Both, MT-1 and MT-2 placental isoforms were separated by HPLC and identified by Western blot (Fig. 1). A typical chromatogram is shown in Fig. 1B. Chromatograms obtained either from placental samples of smokers or non-smokers, showed two main peaks eluting at 6 and 12.5 min and corresponding to MT-1 and MT-2 isoforms, respectively (Fig. 1B). In addition to MT-1 and MT-2 peaks, chromatograms always showed the presence of a smaller peak eluted at 16.5 min, and although we did not identify it, we cannot discard that it may correspond to another MT-related protein. The presence of MT in both peaks was confirmed by Western blot analysis performed in eluants from peaks 1 and 2 using a monoclonal antibody against both MTs (Fig. 1A).

A representative chromatogram of the Cd^{2+} content in fractions eluting from anion exchange HPLC and corresponding to MT-1 and MT-2 is shown in Fig. 2. It can be observed that Cd^{2+} content present in peak 1 (MT-1) from all analyzed samples ($n = 20$) is not significantly different when comparing both groups (median = 44.8 and 53 ng for non-smokers and smokers, respectively).

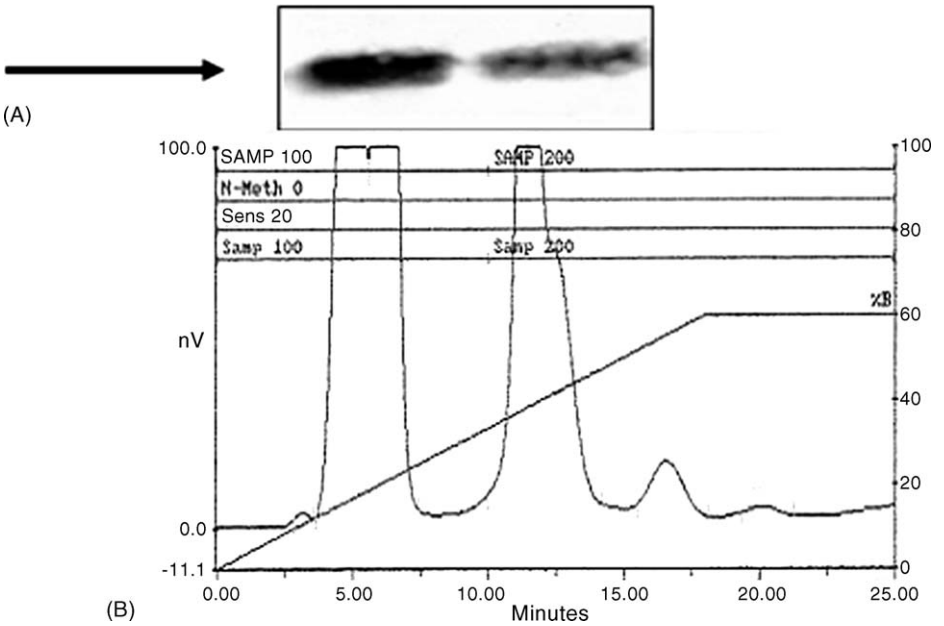

Fig. 1. Separation and identification of MT-1 and MT-2 isoforms from human placenta. (A) MTs Western blot. Eluants from peaks 1 and 2 (B) were freeze-dried, reconstituted in double distilled water and protein concentration determined by the Bradford method. SDS/PAGE was carried out with 20 μg of proteins. After transference of proteins to a PVDF membrane, it was incubated with anti-MT-1/MT-2 antibody as described in Section 2. The arrow shows a band corresponding to MTs contained in peaks 1 and 2. (B) Anion-exchange HPLC separation of human placental MT isoforms was performed with a Fractogel DEAE column (20–40 μm particle size) using a linear gradient from 20 to 200 mM Tris–HCl (0–60% B), pH 7.4 in 25 min at a flow rate of 1 ml/min. A 200 μl aliquot of the prepared sample was injected to the column. The efflux was monitored for UV absorbance at 250 nm.

In contrast, Cd^{2+} content in peak 2 (MT-2) is significantly higher in placentas of smokers (median = 7.4 and 58.1 ng for non-smokers and smokers, respectively; Fig. 2). Total Cd^{2+} concentrations from peaks 1 and 2 were used to calculate MT-1 and MT-2 concentrations according to the equation given in Section 2 (Table 1). Results show that placental MT-1 concentrations present in smokers are not different from those found in non-smokers. However, placental MT-2 concentrations are higher in smokers than in non-smokers; thus, total placental MT concentrations present in smokers are increased by two-fold in comparison to non-smokers (Table 1). Total concentration of Cd^{2+} in the whole placental tissue was also higher in smokers than in non-smokers being 4 and 9 ng (36 and 80 pmol)/g of wet tissue, respectively (Table 2). To estimate the concentration of total MTs bound to Cd^{2+} present in placentas, a maximal binding of 7 mol of Cd^{2+} per mol of MT molecules was considered (Table 2). This

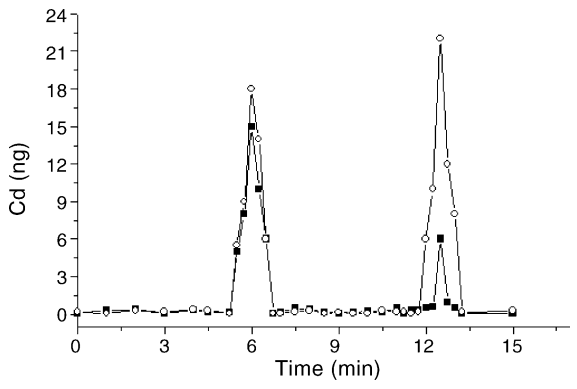

Fig. 2. Representative chromatogram of the elution of Cd^{2+} in MT-1 and MT-2 separated by anion-exchange HPLC. Placental samples saturated with Cd^{2+} were injected on to the anion-exchange column, and fractions were collected at 15 or 30 s intervals. Cd^{2+} content in peaks 1 and 2 (corresponding to MT-1 and MT-2) was determined by AAS-ICP as described in Section 2. (■) Non-smokers; (◯) smokers.

Table 1
MT-1 and MT-2 concentrations in placental samples of non-smokers and smokers

Group	MT-1 (nmol/g wet tissue)	MT-2 (nmol/g wet tissue)	Total MT (nmol/g wet tissue)
Non-smokers[a]	0.90	0.15	1.1
Smokers[a]	1.06	1.16	2.2
Mann–Whitney (*p*)	0.34	0.04	0.05

[a] Median, $n = 10$ placentas. MT-1 and MT-2 concentrations were calculated from the equation given in Section 2 according to the Cd^{2+} content present in the eluants corresponding to peaks 1 and 2.

Table 2
Concentrations of Cd^{2+} in placentas of non-smokers and smokers and estimation of metallothioneins bound and unbound to Cd^{2+}

Group	Total Cd^{2+} (pmol/g wet tissue)[a]	Total MTs (pmol/g wet tissue)[b]	MTs bound to total Cd^{2+} (pmol/g wet tissue)[c]	MTs unbound to Cd^{2+} (pmol/g wet tissue)[d]
Non-smokers	36 (27–46)	1100	5.1	1095
Smokers	80* (62–103)	2200	11.4	2189

[a] Median, $n = 10$ placentas of non-smokers and 10 of smokers. Initial values of 4 and 9 ng of total Cd^{2+} per gram wet tissue corresponding to non-smokers and smokers, respectively, were converted to pmol considering a Cd^{2+} MW of 112.3. Total Cd^{2+} was determined by AAS.
[b] Same values as Table 1 expressed as pmol/g wet placental tissue.
[c] Potential pmol of MTs bound to total Cd^{2+} considering a maximal binding capacity of 7 mol of Cd^{2+} per mol of MT.
[d] Values in [b] minus values in [c].
* $p < 0.05$, Mann–Whitney U-test.

result, which assumes that all MT molecules would be completely saturated with Cd^{2+}, indicates that only 0.5% of total MT is bound to Cd^{2+}. Consequently, as high as 99.5% of total MT are unbound to Cd^{2+} and thus are available to bind other divalent ions, such as Zn^{2+} or Cu^{2+}. It is important to mention that percentage of MT unbound to Cd^{2+} is similar in both; the non-smokers and smokers group (Table 2).

Total placental levels of Zn^{2+} were higher in smokers than in non-smokers being 9.2 and 8.4 μg/g of wet tissue, respectively. These values were converted to nmol (140 and 128 nmol for smokers and non-smokers, respectively; Table 3) to calculate the maximal Zn^{2+} concentrations that could be bound to MT-1 and MT-2 in both groups. These estimations considered a maximal binding capacity of 7 mol of Zn^{2+} per mol of MT-1 or MT-2. Results of Table 3 show that similar concentrations of Zn^{2+} could be potentially bound to MT-1 in both groups. Conversely, higher concentrations of Zn^{2+} could be potentially bound to MT-2 in smokers than in non-smokers. Although Zn^{2+} bound to total MTs may increase by two-folds in smokers, most of placental Zn^{2+} would remain not associated to MTs in both groups since total placental levels of Zn^{2+} are too high in comparison to MT concentrations (Table 3).

4. Discussion

In a recent publication (Ronco et al., 2005a), we reported higher levels of total placental MTs in smokers than in non-smokers. In the present study, we show results demonstrating that the increased MT concentrations are specifically due to the smoking-induced placental MT-2 isoform. This finding suggests that elevated placental MT-2 may be one of the factors involved in the reduced birth weight generally observed in neonates born to smoking mothers.

Metallothioneins are proteins highly conserved through evolution; their presence has been documented throughout the animal kingdom, in higher plants, in eukaryotic organisms and in many prokaryotes (Kägi, 1993; Ridley, 1996). These low molecular weight cysteine-rich proteins belong to a super family of intracellular metal-binding proteins. Their functions include involvement in cell protection against heavy metal toxicity and oxidant damage, and metabolic regulation of Zn^{2+} homeostasis via Zn^{2+} donation, sequestration

Table 3
Concentrations of Zn^{2+} in placentas of non-smokers and smokers and estimation of Zn^{2+} bound and unbound to metallothioneins

Group	Total Zn^{2+} (nmol/g wet tissue)[a]	Zn–MT-1 (nmol/g wet tissue)[b]	Zn–MT-2 (nmol/g wet tissue)[b]	Zn^{2+} bound to total MTs (nmol/g wet tissue)[c]	Zn^{2+} unbound to MTs (nmol/g wet tissue)[d]
Non-smokers	128 (79–179)	6.3	1.05	7.35	120.7
Smokers	140 (111–157)	7.4	8.1	15.5	124.5

[a] Median, $n = 10$ placentas of non-smokers and 10 of smokers. Initial values of 8.4 and 9.2 μg of total Zn^{2+} per gram wet tissue corresponding to non-smokers and smokers, respectively, were converted to nmol considering a Zn^{2+} MW of 65.5. Total Zn^{2+} was measured by INAA.
[b] Potential nmol of Zn^{2+} bound to MT-1 and MT-2 considering a maximal binding capacity of 7 mol of Zn^{2+} per mol of MT-1 or MT-2. Values for MT-1 and MT-2 are those from Table 1.
[c] Zn–MT-1 + Zn–MT-2.
[d] Values in [a] minus values in [c].

and/or redox control (Kang, 1999; Klaassen et al., 1999; Coyle et al., 2002). The most widely expressed isoforms in mammals, MT-1 and MT-2, are rapidly induced in the liver by a wide range of metals, drugs and inflammatory mediators. In other organs, such as the gut and pancreas, MTs respond mainly to Zn^{2+} status (Nath et al., 1988). Metallothionein has also been shown in human placenta and fetal membranes (Wier and Miller, 1987), being mainly located in fetal amniotic cells, syncytial trophoblasts and villous interstitial cells and in maternal decidual cells (Goyer et al., 1992). The presence of MT at those specific sites suggests that it may be implicated in the regulation of transplacental transport of metals such as Zn^{2+}, Cd^{2+} and Cu^{2+}.

It has been recognized that cigarette smoke increases placental levels of Cd^{2+} (Piasek et al., 2001; Ronco et al., 2005a,b). Results obtained in the present study show elevated levels of placental Cd^{2+} and Zn^{2+} in smokers in concordance with previous reports (Kuhnert et al., 1987b).

Therefore, one of the numerous factors involved in the reduced birth weight of infants delivered by smokers could be related to placental Zn^{2+} retention, an effect that may be an indirect consequence of the high levels of Cd^{2+} present in this tissue (Kuhnert et al., 1988b). Maternal MT induction by several chemicals – included toxic heavy metals such as Cd^{2+} – contributes to cause developmental toxicity by a chain of events leading to an adverse decrease in Zn^{2+} bioavailability to the embryo (Daston et al., 1991), a fact which in turn may be involved in low birth weight (Kuhnert et al., 1987b).

In this study, placental MT-1 and MT-2 from smokers and non-smokers were separated by HPLC as previously described for rat liver (Klaassen and Lehman-Mckeeman, 1991). In our experimental protocol, MT-1 and MT-2 retention times were 6 and 12.5 min, respectively, values closely related to rat liver MT-1 and MT-2 retentions times, as already shown by Klaassen and Lehman-Mckeeman (1991), but different to those retention times described by Nostelbacher et al. (2000). Differences may be attributed to different experimental conditions and tissues used. It is important to mention that all the analyzed samples ($n = 20$) showed the same elution profile for both isoforms. The presence of MTs in both elution peaks was corroborated by Western blot analysis. Results demonstrated that in non-smoker's placentas, MT-1 was expressed by six-fold over MT-2 (0.90 nmol/g versus 0.15 nmol/g wet tissue), suggesting different roles for each isoform. Possibly, this MT-1/MT-2 ratio is appropriate for adequate management and regulation of intra-placental Zn^{2+} homeostasis, a condition likely leading to optimal transport of this ion to the fetus. When placental Cd^{2+} levels increase i.e. in smokers, MT-2 levels become similar to MT-1, and as a consequence MT-1/MT-2 ratio changes from six to one. Similar placental MT-1 concentrations are present in non-smokers and smokers (0.9 and 1.06 nmol/g wet tissue, respectively), suggesting that MT-2 is the MT isoform sensitive to increased placental Cd^{2+} levels such as those observed in smokers. This result does not agree with a previous report where the involvement of placental MT-2 in smokers was much lower than MT-1 (Milnerowicz, 1997). Discrepancies are probably due to the different methods used to separate and identify MT-1 and MT-2 isoforms. We used HPLC coupled to Cd^{2+} measurements to separate and quantify MT-1 and MT-2; followed by Western blot analysis for identification. In the study reported by Milnerowicz (1997), identification of MT-1 and MT-2 was based on different electrophoretic mobility in SDS-PAGE, a method which may not be accurate enough to separate and identify different protein isoforms.

Since MTs have high affinity for Cd^{2+} ($K_d = 10^{-17}$ to 10^{-22}) (Klaassen et al., 1999; Coyle et al., 2002) and placental MT concentrations observed are high compared to total Cd^{2+}, all Cd^{2+} ions would be bound to MTs. Furthermore, assuming a maximal binding of 7 mol of Cd^{2+} per mol of MT, most of MTs (99.5% either in smokers or in non-smokers) should remain unbound to Cd^{2+} and thus available to bind other divalent ions. In our case, placental MT-2 resulted elevated in smokers, and as a consequence, the percentage of Zn^{2+} that could be bound to total MTs increased from 5.7% in non-smokers to 11.1% in smokers. Therefore, although Zn^{2+} unbound to MTs is similar in both groups (Table 3), the increased amount of Zn^{2+}–MT-2 complex present in smokers could be relevant enough to interfere with the dynamic of the mechanisms involved in the transference of placental Zn^{2+} to the fetus. This fact could explain, at least in part, the reduced levels of Zn^{2+} previously found in red blood cells of umbilical cord of newborn delivered by smokers (Kuhnert et al., 1987b).

An additional role for the increased placental MT-2 levels found in smokers could be related to fetus protection against Cd^{2+} toxicity. Previous research in non-vertebrate organisms showed that MT-2 expression is the primary response to Cd^{2+} exposure, and established that MT-2 is the sole Cd^{2+}-responsive MT isoform (Sturzenbaum et al., 2001). Furthermore, there is evidence that MT-2 rather than MT-1 is the major isoform implicated in Cd^{2+} detoxification in these organisms (Sturzenbaum et al., 2004). Thus, the elevated levels of MT-2 present in placentas of smokers may have a positive specific function by sequestering and compartmentaliz-

ing Cd^{2+} inside the placenta, avoiding Cd^{2+} transport to the fetus (Boardi et al., 1991).

In summary, results presented in this study show that MT-2 is specifically induced in smoker's placentas, suggesting that this isoform could be involved in placental Cd^{2+} and Zn^{2+} retention. This fact could contribute to reduce the transference of Zn^{2+} to the fetus, which in turn, may be associated to detrimental effects on fetal growth, finally leading to the reduced birth weights observed in neonates born from smoking mothers.

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References

Abel, E.L., 1980. Smoking during pregnancy: a review of effects on growth and development of offspring. Hum. Biol. 52, 593–625.

Atalah, E., Castillo, C., Castro, R., Aldea, A., 1997. Propuesta de un nuevo estándar de evaluación nutricional de embarazadas. Rev. Med. Chile 125, 1429–1436.

Boardi, W.Y., Yannai, S., Urbach, J., Brandes, J.M., Summer, K.H., 1991. Transfer and accumulation of cadmium and the level of metallothionein in perfused human placenta. Arch. Toxicol. 65, 318–323.

Cherian, M.G., Jayasurya, A., Bay, B.H., 2003. Metallothioneins in human tumors and potential roles in carcinogenesis. Mutat. Res. 533, 201–209.

Coyle, P., Philcox, J., Carey, L., Rofe, A., 2002. Metallothionein: the multipurpose protein. Cell. Mol. Life Sci. 59, 627–647.

Daston, G.P., Overmann, G.J., Taubeneck, M.W., Lehman-McKeeman, L.D., Rogers, J.M., Keen, C.L., 1991. The role of metallothionein induction and altered zinc status in maternally mediated developmental toxicity: comparison of the effects of urethane and styrene in rats. Toxicol. Appl. Pharmacol. 110, 450–463.

Davis, S.R., Cousins, R.J., 2000. Metallothionein expression in animals: a physiological perspective on function. J. Nutr. 130, 1085–1088.

Dorea, J.G., 2002. Zn deficiency in nursing infants. J. Am. Coll. Nutr. 21, 84–87.

Ferm, V.H., 1971. Developmental malformation induced by cadmium. Biol. Neonate 19, 101–107.

Goyer, R., Haust, M., Cherian, M., 1992. Cellular localization of MT in human term placenta. Placenta 13, 349–355.

Hamadani, J.D., Fuchs, G.J., Osendarp, S.J., Huda, S.N., Grantham-McGregor, S.M., 2002. Zn supplementation during pregnancy and effects on mental development and behavior of infants: a follow-up study. Lancet 360, 290–294.

Harford, C., Sarkar, B., 1991. Induction of metallothionein by simultaneous administration of cadmium(II) and Zn(II). Biochem. Biophys. Res. Commun. 177, 224–228.

Kägi, J.H.R., 1993. Evolution, structure and chemical activity of class I metallothioneins: an overview. In: Suzuki, K.T., Imura, N., Kimura, M. (Eds.), Metallothionein III: Biological Roles and Medical Implications. Birkhauser, Berlin, pp. 29–56.

Kang, Y.J., 1999. The antioxidant function of metallothionein in the heart. Proc. Soc. Exp. Biol. Med. 222, 263–273.

Klaassen, C., Lehman-Mckeeman, L., 1991. Separation and quantification of isometallothioneins by high-performance liquid chromatography–atomic absorption spectrometry. Methods Enzymol. 205, 190–198.

Klaassen, C., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu. Rev. Pharmacol. Toxicol. 39, 267–294.

Kuhnert, B., Kuhnert, P., Erhard, P., Debanne, S., Williams, T., 1987a. The relationship between cadmium, zinc, and birth weight in pregnant women who smoke. Am. J. Obstet. Gynecol. 157, 1247–1251.

Kuhnert, B., Kuhnert, P., Erhard, P., Brashear, W.T., Groh-Wargo, S.L., Webster, S., 1987b. The effect of smoking on placental and fetal Zn status. Am. J. Obstet. Gynecol. 157, 1241–1246.

Kuhnert, B., Kuhnert, P., Zarlingo, J., 1988a. Associations between placental cadmium and zinc and age and parity in pregnant women who smoke. Obstet. Gynecol. 71, 67–70.

Kuhnert, B.R., Kuhnert, P.M., Lazebnik, N., Erhard, B.S., 1988b. The effect of maternal smoking on the relationship between maternal and fetal Zn status and infant birth weight. J. Am. Coll. Nutr. 7, 309–316.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lehman, L.D., Poisner, A.M., 1984. Induction of metallothionein synthesis in cultured human trophoblasts by cadmium and Zn. J. Toxicol. Environ. Health 14, 419–432.

Liang, L., Fu, K., Lee, D.K., Sobieski, R.J., Dalton, T., Andrews, G.K., 1996. Activation of the complete mouse metallothionein gene locus in the maternal decidium. Mol. Reprod. Dev. 43, 25–37.

Milnerowicz, H., 1997. Influence of tobacco smoking on metallothionein isoforms contents in human placenta, amniotic fluid and milk. Int. J. Occup. Med. Environ. Health 10, 395–403.

Nath, R., Kambadur, R., Gulati, S., Paliwal, V.K., Sharma, M., 1988. Molecular aspects, physiological function and clinical significance of metallothioneins. Crit. Rev. Food Sci. 27, 41–85.

Nostelbacher, K., Kirchgessner, M., Stangl, G., 2000. Separation and quantitation of metallothionein isoforms from liver of untreated rats by ion-exchange high-performance liquid chromatography and atomic absorption spectrometry. J. Chromatogr. B 744, 273–282.

Piasek, M., Blanusa, M., Kostial, K., Laskey, J., 2001. Placental cadmium and progesterone concentrations in cigarette smokers. Reprod. Toxicol. 15, 673–681.

Ridley, M. (Ed.), 1996. Evolution. Blackwell Sci., Cambridge, UK, p. 719.

Ronco, A.M., Arguello, G., Suazo, M., Llanos, M.N., 2005a. Increased levels of metallothionein in placenta of smokers. Toxicology 208, 133–139.

Ronco, A.M., Arguello, G., Muñoz, L., Gras, N., Llanos, M.N., 2005b. Metals content in placentas from moderate cigarette consumers: correlation with newborn birth weight. Biometals 18, 233–241.

Roquer, J., Figueras, J., Botet, F., Jiménez, R., 1995. Influence on fetal growth of exposure to tobacco smoke during pregnancy. Acta Paediatr. 84, 118–121.

Sturzenbaum, S.R., Winters, C., Galay, M., Morgan, A., Kille, P., 2001. Metal ion trafficking in earthworms. J. Biol. Chem. 276, 34013–34018.

Sturzenbaum, S.R., Georgiev, O., Morgan, A.J., Kille, P., 2004. Cadmium detoxification in earthworms: from genes to cells. Environ. Sci. Technol. 38, 6283–6289.

Torreblanca, A., Del Ramo, J., Sarkar, B., 1992. Cadmium effect on zinc metabolism in human trophoblast cells: involvement of cadmium-induced metallothionein. Toxicology 72, 167–174.

Wellinghausen, N., 2001. Immunobiology of gestational Zn deficiency. Br. J. Nutr. 85 (Suppl. 2), S81–S86.

Wier, P.J., Miller, R.K., 1987. The pharmacokinetics of cadmium in the dually perfused human placenta. Trophoblast Res. 2, 357–366.