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RESEARCH PAPER

Role of TNF-α in the mechanisms responsible for preterm delivery induced by Stx2 in rats

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BACKGROUND AND PURPOSE

Infections with a strain of *Escherichia coli* producing Shiga toxins could be one of the causes of fetal morbidity and mortality in pregnant women. We have previously reported that Shiga toxin type 2 (Stx2) induces preterm delivery in pregnant rats. In this study, we evaluate the role of TNF-α, PGs and NO in the Stx2-induced preterm delivery.

EXPERIMENTAL APPROACH

Pregnant rats were treated with Stx2 (0.7 ng g^{-1}) and killed at different times after treatment. Placenta and decidua were used to analyse NOS activity by the conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline, levels of PGE_2 and $PGF_{2\alpha}$ assessed by radioimmunoassay, and cyclooxygenase (COX) proteins by Western blot. TNF-α level was analysed in serum by ELISA and by cytotoxicity in L929 cells. The inhibitor of inducible NOS, aminoguanidine, the COX-2 inhibitor, meloxicam, and the competitive inhibitor of TNF-α, etanercept, were used alone or combined to inhibit NO, PGs and TNF-α production respectively, to prevent Stx2-induced preterm delivery.

KEY RESULTS

Stx2 increased placental PGE_2 and decidual $PGF_{2\alpha}$ levels as well as COX-2 expression in both tissues. Aminoguanidine and meloxicam delayed the preterm delivery time but did not prevent it. Etanercept blocked the TNF-α increase after Stx2 treatment and reduced the preterm delivery by approximately 30%. The combined action of aminoguanidine and etanercept prevented Stx2-induced preterm delivery by roughly 70%.

CONCLUSION AND IMPLICATIONS

Our results demonstrate that the increased TNF-α and NO induced by Stx2 were the predominant factors responsible for preterm delivery in rats.

Abbreviations

Gb3, globotriaosylceramide; gd, gestation day; HUS, haemolytic uremic syndrome; iNOS, inducible nitric oxide synthase; STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin; Stx2, Shiga toxin type 2

Introduction

Gastrointestinal infection with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains causes diarrhoea and haemorrhagic colitis and, in addition, it is the leading cause of the haemolytic uremic syndrome (HUS; Richardson *et al.*, 1988). HUS is characterized by a triad of haemolytic anaemia, thrombocytopenia and acute renal failure (Gianantonio *et al.*, 1964; Repetto, 1997). HUS is most commonly seen in young children, but it is occasionally present in adults (Scully *et al.*, 1995; Repetto, 1997; Frank *et al.*, 2011), including *post partum* women. Most of the *post partum* cases had preceding

upper respiratory or gastrointestinal symptoms (Strauss and Alexander, 1976; Steele *et al.*, 1984) and at least in one case, HUS was detected after a preterm delivery at 32 weeks (Steele *et al.*, 1984). However, to our knowledge, an increased risk of preterm delivery linked to STEC infection has not yet been evaluated.

HUS is a systemic complication attributed to the expression of Stx (Karmali *et al.*, 1983). Two types of Stx may be produced by STEC strains: Stx1 and/or Stx2 with their variants (Paton and Paton, 1998). In Argentina, STEC strain O157 : H7 producing only Stx2 is highly prevalent (Rivas *et al.*, 2006). Stx is an AB5 holotoxin possessing a single A subunit in non-covalent association with five B subunits (Fraser *et al.*, 1994). The B subunits form a pentameric ring and mediate toxin binding to the glycolipid receptor globotriaosylceramide (Gb3) expressed in vascular endothelial cells, podocytes, mesangial cells and epithelial cells of renal proximal tubules (Lingwood *et al.*, 1987; Lingwood, 1996). The A subunit is an N-glycosidase, which removes an adenine of the 28S ribosomal RNA resulting in cell death (Endo *et al.*, 1988).

We have previously reported that i.p. administration of Stx2 in the late stage of pregnancy induces preterm delivery of dead fetuses (Burdet *et al.*, 2009). Overproduction of NO and damage in placenta which was prevented by aminoguanidine, an inhibitor of inducible NOS (iNOS), demonstrated that NO plays an important role in placental toxicity and fetal mortality induced by Stx2 (Burdet *et al.*, 2010).

During inflammation, PGs are released simultaneously with NO and their overproduction could be detrimental. PGs promote uterine contractions contributing to embryonic expulsion (Aisemberg *et al.*, 2007). Stx may act in concert with bacterial lipopolysaccharide (LPS) and induce the production of TNF-α, IL-1 and IL-6 by macrophages rendering the vascular endothelial cells are more sensitive to the toxin (Louise and Obrig, 1991; Tesh *et al.*, 1994). Furthermore, high concentrations of many cytokines in the vaginal or cervical secretions, including TNF-α and IL-6 in women with symptoms of preterm labour, are associated with early preterm delivery (Inglis *et al.*, 1994).

These observations led us to investigate whether PGs and TNF-α could be involved in Stx2-induced preterm delivery in rats.

Methods

Animals

All animal care and experimental procedures in this study were in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved by the Committee for the Care and Use of Laboratory Animals of the University of Buenos Aires (CICUAL, Permit Number 1209–10). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of N animals were used in the experiments described here. The animals received food and water *ad libitum* and were housed under controlled conditions of light (12 h light, 1 h dark) and temperature (23–25°C). To obtain timed pregnant females, male and virgin female Sprague-Dawley rats between 250 and 300 g of body weight (bwt) were acquired from the animal facility of the School of Veterinary, University of Buenos Aires. Mating was performed placing the female rats in the cages of the male rats from the same strain for several days. Day 1 of gestation was determined when sperm was observed in the vaginal smear.

Experimental protocols

Pregnant rats on day 15 of gestation (gd) were randomly divided into groups of at least three rats each. The Stx2 injury was induced as previously described (Burdet *et al.*, 2009). Briefly, Stx2-treated rats were injected (i.p.) with 0.5 mL of culture supernatant from recombinant *E. coli,* equal to a dose of 0.7 ng Stx2 and 50 pg LPS per gram. Control rats were inoculated with 0.5 mL of culture supernatant containing only LPS (50 pg LPS g^{-1}). In separate experiments, control and Stx2-treated rats were injected (i.p.) with aminoguanidine (100 μg g^{-1}) 24 h before and 4 h after toxin injection; meloxicam (2 μg g^{-1}) simultaneously with the toxins and 12, 24 and 36 h later; etanercept (100 μg g^{-1}) 6 h before toxins; a combination of aminoguanidine and meloxicam, or aminoguanidine and etanercept according the protocol previously described for each one. Rats from the different experimental groups were observed daily to evaluate delivery time and fetal status. Some of them were anaesthetized and killed by cervical dislocation at different times after treatment. Placenta and decidua tissues were removed to evaluate PGs synthesis, NOS activity, COX-1 and COX-2 protein expression. Serum and amniotic fluid were obtained to measure TNF-α production.

Determination of PGs

Placenta and decidua were used to measure PGE_2 and $PGF_{2\alpha}$ levels as previously detailed (Ribeiro *et al.*, 2005). Briefly, tissues were incubated for 1 h in Krebs-Ringer bicarbonate solution under a 5% CO_2 atmosphere at 37°C. Afterwards, medium was acidified and PGs were extracted twice with ethyl acetate. PG concentration was determined by radioimmunoassay. Sensitivity was 5–10 pg per tube and values are expressed as pg PG (mg protein)$^{-1}$.

Determination of NOS activity

NOS enzyme activity in placenta was quantified by measuring the conversion of L-[^{14}C]arginine into L[^{14}C]citrulline as described before (Burdet *et al.*, 2010). Samples were weighed, homogenized and incubated with 10 μM L-[^{14}C]arginine (0.3 μCi). After 15 min, samples were centrifuged for 10 min at 10 000 × *g* and applied to a Dowex AG50-X8 column. L-[^{14}C]citrulline was eluted and measured by liquid scintillation counting. Enzyme activity is reported as fmol L-[^{14}C]citrulline formed (mg protein)$^{-1}$ over 15 min.

TNF-α bioassay and immunoassay

TNF bioactivity in serum and amniotic fluid samples was assayed employing the TNF-sensitive cell line L929 (Shiau *et al.*, 2001). In brief, 1×10^4 L929 cells per well were seeded into a 96-well tissue culture plate and cultured in RPMI 1640 medium supplemented with 5 μg mL^{-1} streptomycin, 5 U mL^{-1} penicillin and 10% fetal calf serum. The medium was replaced by 0.1 mL of medium alone, or with samples

from control or Stx2-treated rats. The L929 cells were grown in arrested-growth conditions (actinomycin D, 10 μg mL^{-1}). After 24 h incubation, the cells were washed with PBS and viability was assayed by using the neutral red uptake technique (Goldstein *et al.*, 2007). Bioactive TNF-α was expressed as percentage of L929 cell viability. Concentrations of TNF-α were measured with the Biotrak rat TNF-α ELISA system according to the manufacturer's instructions.

Western blot analysis

Tissues were processed according to the method described by Burdet *et al.*, (2010). In brief, placental and decidual tissues were fragmented and homogenized on ice in an appropriate buffer with protease inhibitors. Homogenates were pre-centrifuged at 2500 × *g* for 10 min at 4°C and the collected supernatants were additionally centrifuged at 7800 × *g* for 10 min at 8°C. The supernatants were collected and stored at –70°C until Western blotting was performed. Protein concentrations were determined with the BCA Protein Assay Kit. Equal amounts of protein (100 μg) were loaded in each line. Samples were separated on 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel by electrophoresis and transferred to a PVDF membrane. The blots were incubated 48 h at 4°C with anti-COX-1 or anti-COX-2 rabbit polyclonal antibody diluted 1:250 in PBS. The membranes were then incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG antibody (1:3000). Proteins were detected through ECL detection system. To determine the uniformity of loading, protein blots were probed with a monoclonal anti-β-actin antibody (1:4000). Band intensities were measured using the Quantity One densitometry software package (Bio-Rad Lab, USA). Protein bands were normalized to their respective β-actin bands.

Data analysis

Statistical analysis was performed using the Graph Pad Prism Software (San Diego, CA, USA). Comparison between values of different groups was performed using one-way ANOVA. Significance was determined using Tukey's multiple comparison test for unequal replications, or Student's *t*-test. Fisher exact test was used to compare the term and preterm delivery under different treatments. Statistical significance was set at $P < 0.05$.

Materials

L-[^{14}C]arginine (specific activity 360 mCi $mmol^{-1}$), [5,6,8,11,12,14,15-^{3}H(N)]- PGE_2 (specific activity 150 Ci $mmol^{-1}$) and [5,6,8,9,11,12,14,15-^{3}H(N)]-$PGF_{2\alpha}$ (specific activity 200 Ci $mmol^{-1}$) were from Perkin Elmer Life and Analytical Sciences, Walthman, MA, USA. ECL detection system, Biotrak rat TNF-α ELISA and actinomycin D were from GE Healthcare Life Sciences, Piscataway, NJ, USA. Meloxicam was purchased from Boehringer Ingelheim, Mainz Bingen, Germany. Aminoguanidine , PGE_2 and $PGF_{2\alpha}$ standards, monoclonal β-actin antibody, secondary antibodies, Luria-Bertani broth and ampicillin were from Sigma Chemical Corp, St Louis, MO, USA. Etanercept was from Wyeth Lab, Madison, NJ, USA. The Dowex AG50-X8 column (Na^+ form), nitrocellulose membranes and all other Western blot reagents were from Bio-Rad Lab, Hercules, CA, USA. Filters were from Millipore Corp, Billerica, MA, USA. Primary COX antibodies were from Cayman Chemical, Ellsworth Road, MI, USA. The HEK-Blue LPS Detection Kit was from InvivoGen, San Diego, CA, USA. The BCA Protein Assay Kit was from Pierce Biotechnology Inc, Rockford, IL, USA. All other chemicals were analytical grade.

Results

Stx2 increases PGs levels in placenta and decidua

Taking into account that during inflammation, PGs are released simultaneously with NO, we examined PGE_2 and $PGF_{2\alpha}$ formation in placenta and decidua from rats treated with Stx2 alone or in the presence of aminoguanidine. In placenta, PGE_2 showed an increase 12 h after Stx2 injection ($P < 0.01$, $n = 18$) while $PGF_{2\alpha}$ remained constant. Treatment with aminoguanidine plus Stx2 caused a greater increase in PGE_2 ($P < 0.001$, $n = 18$) than the increase detected only with Stx2 and also caused an increase in $PGF_{2\alpha}$ ($P < 0.01$, $n = 18$; Figure 1A). In decidua, PGE_2 decreased and $PGF_{2\alpha}$ increased

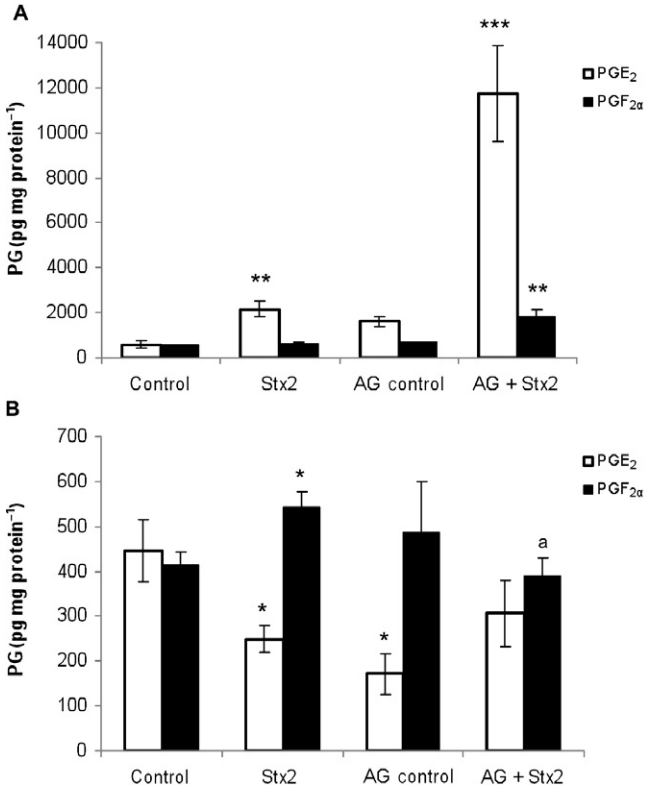

Figure 1

Effects of Stx2 and aminoguanidine (AG) on PG production. Pregnant rats (gd 15) were treated with LPS alone (50 pg g^{-1}) (shown as control) or with 0.7 ng Stx2 and 50 pg LPS g^{-1} (shown as Stx2) and killed 12 h post-treatment. Some animals were injected with aminoguanidine (i.p., 100 μg g^{-1}) 24 h before and 4 h after toxin injection. PGE_2 and $PGF_{2\alpha}$ levels were evaluated in placenta (A) and decidua (B) by RIA. Data corresponding to at least two separate experiments are shown. Values are expressed as means ± SEM ($n = 18$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control; $^{a}P < 0.05$ versus Stx2.

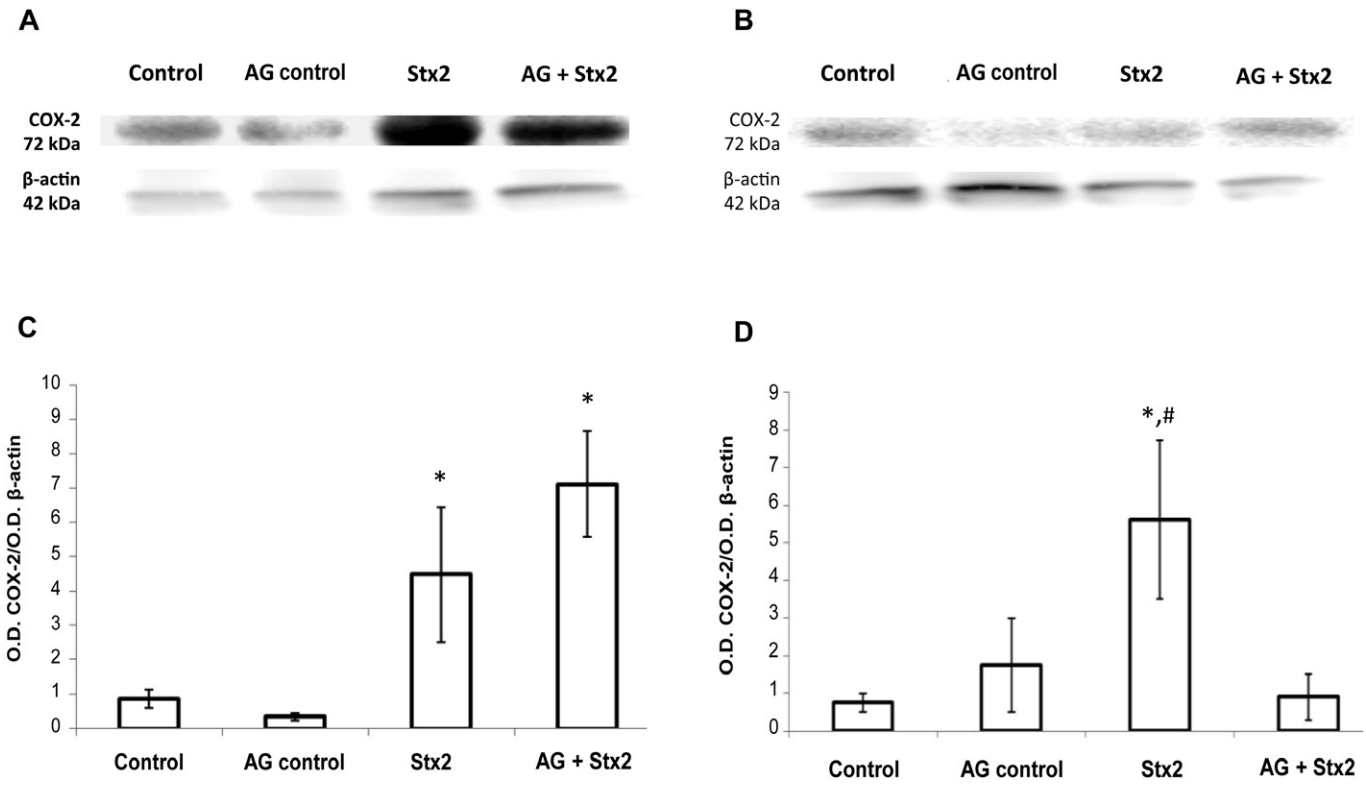

Figure 2

Effect of Stx2 and aminoguanidine (AG) on COX-2 protein expression. Pregnant rats were injected with LPS alone (50 pg g^{-1}) (shown as control) or with 0.7 ng Stx2 and 50 pg LPS g^{-1} (shown as Stx2) and killed 12 h post-treatment. Some animals were injected with aminoguanidine (i.p., 100 μg g^{-1}) 24 h before and 4 h after toxin treatment. Placenta and deciduas were removed for Western blot analysis. To determine the uniformity of loading, blots were probed with a monoclonal anti-β-actin antibody. Representative gel for COX-2 protein expression in placenta (A) and decidua (B) and the corresponding densitometry of the bands (C and D, respectively) are shown. Values correspond to the mean of two different pools of six animals ± SEM. *$P < 0.05$ versus control; $^{\#}P < 0.05$ versus Stx2 + aminoguanidine .

($P < 0.05$, $n = 18$) in Stx2-treated rats while both PGs were similar to the controls after aminoguanidine plus Stx2 treatment (Figure 1B).

Stx2 *stimulates the expression of COX-2 in placenta and decidua*

An increase in COX-2 protein expression was detected in placenta from rats treated with Stx2 alone or in the presence of aminoguanidine (Figure 2A and C, $P < 0.05$, $n = 6$). An increase in COX-2 expression was also detected in decidua from Stx2-treated rats ($P < 0.05$, $n = 6$) although the expression was similar to the controls after aminoguanidine treatment (Figure 2B and D). None of the applied treatments changed the expression level of COX-1 in placental and decidual tissues of Stx2-treated rats (data not shown). These results suggest that changes in the levels of PGs caused by Stx2 were mediated by COX-2 and modulated by NO production.

Aminoguanidine and meloxicam delay the preterm delivery induced by Stx2

In order to determine whether the alterations in the PG levels detected in placenta and decidua from Stx2-treated rats may be in part responsible for preterm delivery, meloxicam, a selective inhibitor of COX-2, was used. Table 1 shows that meloxicam did not prevent preterm delivery in Stx2-treated rats. All the rats treated with meloxicam and Stx2 had premature delivery of dead fetuses while control rats treated with meloxicam delivered normal live pups at term (Table 1, row 4 vs. 3). Furthermore, we studied the ability of aminoguanidine combined with meloxicam to prevent Stx2-induced preterm delivery. Previously, we have showed that aminoguanidine administration did not prevent the premature delivery of dead fetuses (Table 1, row 6; Burdet *et al.*, 2010). Instead, co-administration of aminoguanidine and meloxicam delayed preterm delivery by around 2 days in 50% of the rats, 3 days in 17% of the rats and 4 days in 33% of the rats but did not prevent the preterm delivery of dead fetuses (Table 1, row 8). These results suggest that both NO and PGs are involved in the action of Stx2 on pregnant rats, although their blockade was not enough to reverse the preterm delivery induced by Stx2.

Stx2 induces deregulation of serum TNF-α production

TNF-α is one of the main cytokines mediating the inflammatory response to Stx2 and its deregulation, either systemic or

Table 1

Effect of meloxicam (Melo), aminoguanidine (AG) and etanercept (ETA) on delivery time and pups status

Treatment	*n	Preterm delivery (%)					Term delivery (%)		Live/dead pups (%)
		gd 16–17	gd 18	gd 19	gd 20	gd 21	gd 22	gd 23	
Control	6	0	0	0	0	0	0	100	100/0
Stx2	8	100	0	0	0	0	0	0[a]	0/100
†Melo control	6	0	0	0	0	0	0	100	100/0
†Melo + Stx2	8	100	0	0	0	0	0	0[b]	0/100
#AG control	6	0	0	0	0	0	0	100	100/0
#AG + Stx2	6	100	0	0	0	0	0	0[c]	0/100
#AG+ †Melo control	6	0	0	0	0	0	0	100	100/0
#AG+ †Melo + Stx2	6	0	0	50	17	33	0	0[d]	0/100
¥ETA control	3	0	0	0	0	0	0	100	100/0
¥ETA + Stx2	10	30	30	0	0	10	0	30	30/70
#AG + ¥ETA control	3	0	0	0	0	0	0	100	100/0
#AG + ¥ETA + Stx2	10	0	0	0	0	30	0	70[e]	70/30

†Meloxicam: 2 μg g^{-1}; #aminoguanidine 100 μg g^{-1}; ¥etanercept 100 μg g^{-1}. *n = number of rats.
[a]$P < 0.005$ versus control. [b]$P < 0.005$ versus meloxicam control. [c]$P < 0.005$ versus aminoguanidine control. [d]$P < 0.005$ versus aminoguanidine + meloxicam control. [e]$P < 0.005$ versus Stx2. [a,b,c,d,e]Calculated by Fisher exact test.

locally, could contribute to the changes observed during the progress of pregnancy. To test this hypothesis, we determined the TNF-α levels in serum and amniotic fluid of Stx2-treated rats as well as its toxicity on the TNF-sensitive cell line L929.

An increase in the level of TNF-α protein in serum of Stx2-treated rats was maximal at 2 h after Stx2 injection, after which levels returned to normal at 6 h and 12 h (Figure 3A). Consequently, the toxic activity of TNF-α showed a reduction in the viability of L929 cells to approximately 47% at 2 h after Stx2 injection (Figure 3B). However, the TNF-α level did not change in the amniotic fluid of pregnant rats treated with Stx2 (data no shown).

Pretreatment with etanercept prevented the increase of TNF-α production in serum samples induced by Stx2 (Figure 4). Etanercept also prevented the increase in placental PGE_2 ($P < 0.001$, $n = 18$) and decidual $PGF_{2\alpha}$ ($P < 0.001$, $n = 18$) levels caused by Stx2 (Figure 5A and B). In contrast, no significant difference in placental NOS activity was found in rats treated with etanercept + Stx2 compared with Stx2 alone. In both cases, a significant increase in NOS activity in placental tissues ($P < 0.01$, $n = 6$) was observed, compared with the control (Figure 6).

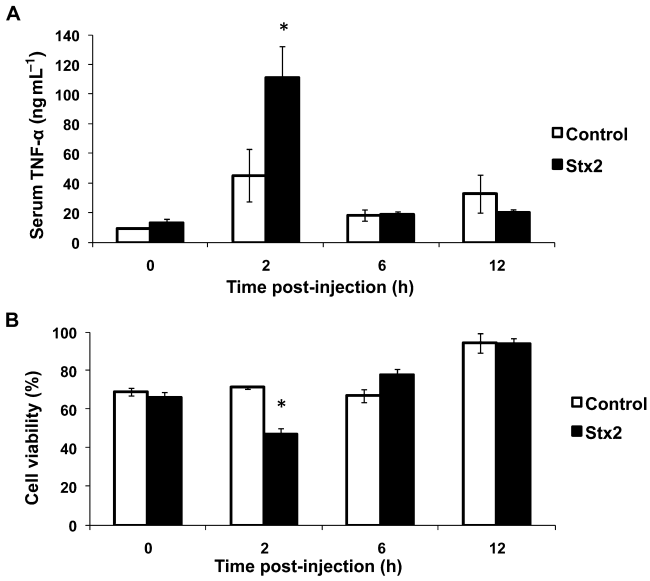

Figure 3

TNF-α production in pregnant rats. Pregnant rats were injected with LPS alone (50 pg g^{-1}) (shown as control) or with 0.7 ng Stx2 and 50 pg LPS g^{-1} (shown as Stx2) and killed 0, 2, 6 and 12 h after the injection. Serum samples were used to determine amount of TNF-α by ELISA assay (A) and TNF-α cytotoxic activity by viability assay in L929 cells (B). Data corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$ versus control.

Etanercept and aminoguanidine prevent the preterm delivery induced by Stx2

To determine whether the increase in TNF-α production and NOS activity triggers the Stx2-induced preterm delivery, we evaluated the action of etanercept on the delivery time and fetal status of Stx2-treated rats. Administration of etanercept 6 h before Stx2 injection prevented the preterm delivery by roughly 30% compared with their controls (Table 1 row 10 vs. 9). Furthermore, we evaluated the combined action of aminoguanidine and etanercept in Stx2-treated rats compared with control rats. For the first time, we observed that the treatment with both inhibitors, aminoguanidine plus etanercept, significantly ($P < 0.005$, $n = 10$) prevented Stx2-induced preterm delivery in 70% of the cases (Table 1, row 12). The pups of these rats were born alive and had normal development com-

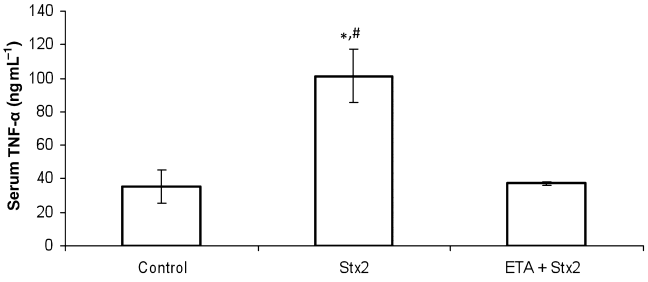

Figure 4

Effects of etanercept (ETA) on TNF-α production. Pregnant rats were injected with LPS alone (50 pg g^{-1}) (shown as control) or with 0.7 ng Stx2 and 50 pg LPS g^{-1} (shown as Stx2) and killed 2 h after injection. Some animals were injected with etanercept (i.p., 100 μg g^{-1}) 6 h before toxin treatment. Serum samples were used to determine amount of TNF-α by ELISA assay. Data corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM (n = 4). *P < 0.05 versus control, #P < 0.05 versus etanercept + Stx2.

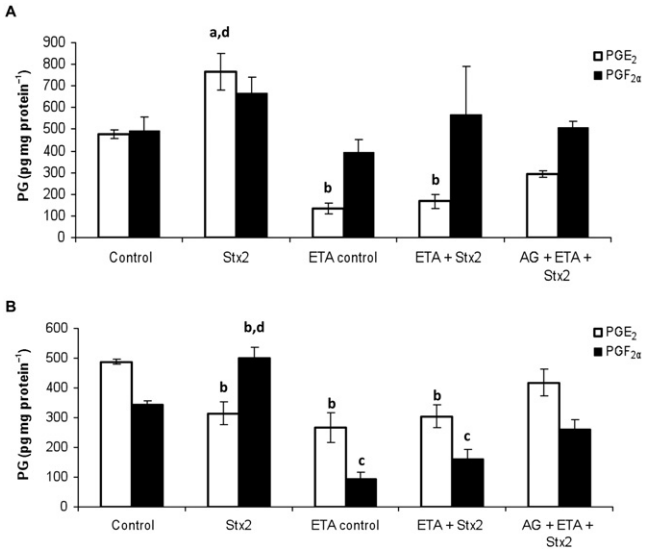

Figure 5

Effects of etanercept (ETA) and aminoguanidine (AG) on PG production. Pregnant rats (gd 15) were injected with LPS alone (50 pg g^{-1}) (shown as control) or with 0.7 ng Stx2 and 50 pg LPS g^{-1} (shown as Stx2) and killed 12 h post-injection. Some animals were injected with etanercept (i.p., 100 μg g^{-1}) 6 h before toxin treatment. Others were treated with a combination of aminoguanidine and etanercept according the protocol described before for each one. PGE_2 and $PGF_{2\alpha}$ levels were evaluated in placenta (A) and decidua (B) by RIA. Data corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM (n = 18). [a]P < 0.05 versus control, [b]P < 0.01 versus control, [c]P < 0.001 versus control, [d]P <0.001 versus etanercept control, etanercept + Stx2 and aminoguanidine + etanercept + Stx2.

pared with their controls (Table 1, row 11). These results suggest that overproduction of TNF-α and NO are the major factors responsible for the preterm delivery of dead fetuses induced by Stx2.

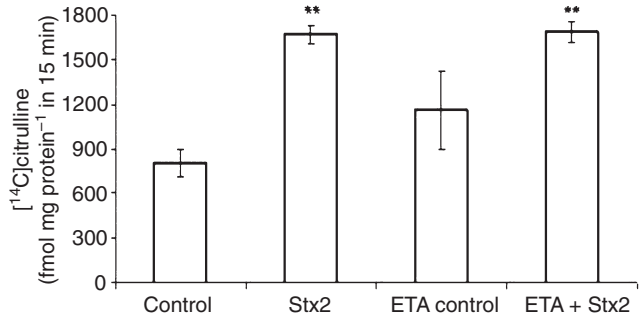

Figure 6

Effect of etanercept (ETA) on NOS activity. Pregnant rats (gd 15) were injected with LPS alone (50 pg g^{-1}) (shown as control) or with 0.7 ng Stx2 and 50 pg LPS g^{-1} (shown as Stx2) and killed 12 h post-injection. Some animals were treated with etanercept (i.p., 100 μg g^{-1}) 6 h before toxin treatment. NOS activity in placenta was quantified by measuring the conversion of L-[^{14}C]arginine to L-[^{14}C] citrulline. Data corresponding to at least two separate experiments are shown. Values are expressed as the mean ± SEM (n = 6). **P < 0.01 versus control.

Discussion

In this study, a significant increase in placental PGE_2 and decidual $PGF_{2\alpha}$ was found in Stx-treated rats. Additionally, in both tissues, COX-2 protein expression was stimulated by Stx2, although meloxicam, a specific inhibitor of COX-2, was unable to prevent placental toxicity and fetal mortality. Here, we found that production of PGs and expression of COX-2 protein were modulated by NOS activity in Stx2-treated rats. Thus, it is reasonable to infer that NO could elicit different effects on the progression of pregnancy, some directly related with its overproduction and others related with the regulation of PG production (Aisemberg *et al.*, 2007). We reported before that aminoguanidine prevented placental damage in Stx2-treated rats but did not prevent preterm delivery (Burdet *et al.*, 2010). Here, the administration of aminoguanidine, together with meloxicam, delayed preterm delivery but did not prevent it, which indicates that other factors are involved in the Stx2-induced preterm delivery. It is well known that Stx may act in concert with LPS to elicit cellular dysfunction (Louise and Obrig, 1992). Stx is able to bind to Gb3 receptors expressed on the membrane of monocytes/macrophages and leads to cellular activation and secretion of cytokines such as TNF-α, IL-1β and IL-8 that increase endothelial susceptibility to Stx (Ibarra and Palermo, 2010). Many studies conducted in human and experimental animals have established that a correct balance of cytokines at the maternal-fetal interface is an essential requirement for proper placental development and therefore reproductive success (Peltier, 2003; Gravett *et al.*, 2007). In pregnant rats, administration of TNF-α produced placental injury and fetal death similar to the effects observed after LPS exposure (Silen *et al.*, 1989). In this study, we showed that a significant increase in serum TNF-α observed 2 h after Stx2 treatment may be responsible for preterm delivery. Pregnancy loss was triggered by abnormal inflammation as reported by other researchers (Grigsby *et al.*,

2003; Renaud *et al.*, 2011). TNF-α in amniotic fluid was not detected suggesting that maternal, more than fetal, TNF-α exerted its detrimental effects on pregnancy. Administration of etanercept completely prevented the increase in serum TNF-α level caused by Stx2 as previously demonstrated in astrocytes treated with LPS and Stx1 (Landoni *et al.*, 2010). Moreover, etanercept prevented by 30% of the preterm delivery caused by Stx2 and, although this was not significant, it showed a clear trend towards prevention of the effects of Stx2. Our results indicate that TNF-α may play a causal role in Stx2 pregnancy loss, consistent with previous studies reported in human and animal models (Silver *et al.*, 1994; Raghupathy *et al.*, 2000). Etanercept also inhibited the induction of placental PGE_2 and decidual $PGF_{2\alpha}$ synthesis in Stx2-treated rats, even in the presence of aminoguanidine. These findings indicate that TNF-α stimulates PG production independent of NO production as already described (Romero *et al.*, 1988; Sato *et al.*, 2003). Etanercept did not inhibit the increase of placental NOS activity caused by Stx2. Thus, it is reasonable to infer that Stx2-induced increase in NO production is not mediated by TNF-α. We assayed the combined action of aminoguanidine and etanercept on fetal status and delivery time in Stx2-treated rats. Co-administration of aminoguanidine and etanercept significantly prevented preterm delivery caused by Stx2 in approximately 70% of the cases. The pups of these rats were similar in size and weight to those observed in the controls. These results indicate that the preterm delivery of dead fetuses induced by Stx2 is triggered by TNF-α and mediated by an increase in NOS production. Both placental PGE_2 and decidual $PGF_{2\alpha}$ increase secondarily to the increase in TNF-α and output of iNOS and contribute to the mechanisms that lead to the preterm delivery.

In conclusion, the model presented in this study is relevant because it shows that Stx2 increases TNF-α production, which renders the feto-maternal unit more susceptible to Stx2 through the stimulation of local PG synthesis, which in turn may activate uterine contractions and cervical dilation. This proinflammatory environment could promote the contraction of the uterus and the expulsion of the fetuses. Additionally, Stx2 induces the overproduction of NO as a consequence of an increase in the levels of iNOS protein in placental tissues, which plays an important role in placental toxicity and fetal mortality.

It is important to note that the administration of aminoguanidine and etanercept partially prevented preterm delivery of dead fetuses induced by Stx2. This suggests that they could be used in the future as therapeutic agents in this pathological consequence of HUS.

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Conflict of interest

None.

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