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Lack of effect of proteinase-activated receptor-2 (PAR-2) deletion on the pathophysiological changes produced by lipopolysaccharide in the mouse: comparison with dexamethasone

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Abstract

This study tested the hypothesis that activation of proteinase-activated receptor-2 (PAR-2) contributes towards the pathophysiology of lipopolysaccharide (LPS)-induced shock in the mouse. The effects of LPS on plasma glucose, biochemical markers of hepatic, renal and pancreatic exocrine function and lung content of myeloperoxidase (MPO) were examined in homozygous PAR-2 knockout mice (PAR-2 –/–) and genetically equivalent, homozygous PAR-2 +/+ mice. The effect of LPS was also examined in normal mice receiving dexamethasone (10 mg kg^{-1}, i.p.) or saline as a positive control. At six hours after intraperitoneal injection, LPS (40 mg kg^{-1}) produced an increase in rectal temperature, hypoglycaemia and elevations in serum concentrations of alanine aminotransferase (ALT), creatinine and lipase, as well as an increase in lung MPO content. Dexamethasone treatment reduced LPS-induced hypoglycaemia and elevation of serum ALT concentrations but did not modify elevations in serum creatinine and lipase concentrations or the increase in lung MPO content. The changes in serum concentrations of glucose, ALT, creatinine and lipase produced by LPS in PAR-2 –/– mice were not different from those seen in wild-type or PAR-2 +/+ mice. These data suggest that activation of PAR-2 may not play a pivotal role in LPS-induced multi-organ dysfunction.

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Introduction

Proteinase-activated receptors (PARs) comprise a family of G-protein-coupled receptors containing four members (PAR-1, -2, -3, and -4) with a unique mode of activation through cleavage of the receptor by serine proteases (Macfarlane et al 2001). There is growing evidence for the involvement of PAR-2 receptors in inflammation. Administration of selective PAR-2 agonists produces inflammation in rat paws and the receptor is expressed on endothelial cells (Vergnolle et al 1999a; Hamilton et al 2001) and infiltrating white cells (Vergnolle et al 1999a) in inflamed tissue. Activation of this receptor promotes leucocyte rolling, adhesion and extravasation (Vergnolle 1999b; Lindner et al 2000). The onset of leucocyte rolling in response to surgical trauma was delayed slightly but significantly in PAR-2-deficient mice, although the leucocytic recruitment following induction of peritonitis was not significantly different (Lindner et al 2000). However, adjuvant arthritis in PAR-2 knockout mice was markedly reduced compared with that seen in wild-type mice (Ferrell et al 2003) and, compared with wild-type mice, eosinophil infiltration during allergic inflammation of the airway was inhibited by 73% in mice lacking PAR-2 (Schmidlin et al 2002). There is evidence that stimulation of the PAR-2 receptor can activate the NFκB (nuclear factor kappa B) pathway (Kanke et al 2001), which also plays a key role in mediating the effects of bacterial lipopolysaccharide (LPS) (Muller et al 1993). Exposure of human endothelial cells to LPS produced a similar increase in PAR-2 expression to that produced by cytokines (Nystedt et al 1996). The PAR-2 agonist peptide SLIGKV amplified the effect of LPS in increasing interleukin-6 (IL-6) production in human umbilical vein endothelial cells and this amplification was associated with a

corresponding increase in nuclear NFκB proteins (Chi et al 2001). Moreover, hypotension induced by the PAR-2 receptor ligand SLIGRL was enhanced in LPS-treated rats (Cicala et al 1999). Thus it may be hypothesised that PAR-2 plays a role in lipopolysaccharide (LPS)-induced shock. On the other hand, the PAR-2 ligand SLIGRL markedly inhibited LPS-induced neutrophil influx and elevation in matrix metalloprotease activity in the airways in response to intranasal administration of LPS in mice (Moffatt et al 2002). Thus, the role of PAR-2 in the pathophysiology of endotoxaemia is unclear and there are currently no selective PAR-2 blocking agents with which to examine this role. Therefore, to test the hypothesis that PAR-2 contributes to shock induced by LPS, we used mice lacking PAR-2 and examined the effects of LPS on serum concentrations of glucose, alanine aminotransferase (ALT, as an indicator of hepatic dysfunction), creatinine (as an indicator of renal dysfunction), lipase (as an indicator of exocrine pancreatic dysfunction) and lung myeloperoxidase, as an indication of pulmonary white cell infiltration.

Materials and Methods

Drugs

Dexamethasone sodium phosphate was obtained from Sigma-Aldrich Company (Fancy Road, Poole, Dorset, UK) and *Eschericia coli* lipopolysaccharide (055:B5) was obtained from Difco laboratories.

Mice

The experiments were performed under the Animals (Scientific Procedures) Act of 1986. PAR-2 knockout mice were generated as outlined previously using a standard homologous recombination strategy (Ferrell et al 2003). Briefly, an 813-nucleotide internal region of exon-2 was removed and replaced with the reporter/selection cassette TAG3/IRES/LacZpa/MC1neopA and used to generate appropriate ES cell clones. From these ES cells mouse chimeras were generated and homozygous PAR-2 $-/-$ mice obtained from initial PAR-2 $+/-$ test cross offspring. The PAR-2 $-/-$ mice were backcrossed onto a C57Bl/6 background over five generations and equivalent homozygous PAR-2 $+/+$ mice were established. Southern blot analysis of tail DNA was used to confirm the genetic identity of the mice.

Mice, 22–25 g, were treated with *E. coli* LPS ($40\,mg\,kg^{-1}$, i.p.) or with pyrogen free saline ($1\,mL\,kg^{-1}$, i.p.). The LPS dose was based on preliminary experiments which showed that, in this strain of mouse, this dose produced more consistent hypoglycaemia and elevations in serum concentrations of ALT, creatinine and lipase and lung content of myeloperoxidase (MPO) than did lower doses (25 or $12.5\,mg\,kg^{-1}$), although mortality (0–40%) was low over the 6-h duration of these experiments. Two hours later the mice were anaesthetised with pentobarbital sodium ($90\,mg\,kg^{-1}$, i.p.). The ambient temperature was maintained at $\sim$30°C using a lamp placed over the cages, to prevent the profound hypothermia that was found in preliminary experiments in pentobarbital-anaesthetised mice maintained at normal room temperature. At 6 h after injection, the anaesthesia was supplemented with 3% isoflurane in oxygen, the abdomen was opened and up to 0.75 mL blood obtained from the inferior vena cava. The blood was immediately chilled, allowed to clot and then centrifuged to separate the serum, which was stored for subsequent assay of glucose, ALT, creatinine and lipase. One lung was removed and assayed for MPO. All mice received isotonic (0.9% w/v sodium chloride) saline ($10\,mL\,kg^{-1}$) at time zero (i.p. with LPS), 2 h (i.p. with pentobarbital anaesthetic) and at 4 h (s.c.).

Biochemical determinations

Serum concentrations of ALT, creatinine and lipase were measured using standard kits obtained from Sigma. Serum concentrations of glucose were measured using a Beckman Glucose Analyzer. Lung MPO was measured by the method of Bradley et al (1982).

Experimental designs

Effect of PAR-2 deletion

Four treatment groups ($n = 8$) were used in parallel, the different mice being treated in a randomised order: saline ($1\,mL\,kg^{-1}$, i.p.) given to $+/+$ mice; LPS ($40\,mg\,kg^{-1}$, i.p.) given to $+/+$ mice; saline ($1\,mL\,kg^{-1}$, i.p.) given to $-/-$ mice; LPS ($1\,mL\,kg^{-1}$, i.p.) given to $-/-$ mice.

Effect of dexamethasone

An additional experimental series was undertaken in which dexamethasone was used as a positive control agent. Dexamethasone ($10\,mg\,kg^{-1}$, i.p.) or saline ($1\,mL\,kg^{-1}$, i.p.) was injected 30 min before LPS. This study used a randomised design in which mice were allocated to one of 4 treatment groups studied in parallel (built up to $n = 8$ across two days): saline ($1\,mL\,kg^{-1}$, i.p.) followed 30 min later by saline ($1\,mL\,kg^{-1}$, i.p.); saline ($10\,mL\,kg^{-1}$, i.p.) followed 30 min later by LPS ($40\,mg\,kg^{-1}$, i.p.); dexamethasone ($10\,mg\,kg^{-1}$, i.p.) followed 30 min later by saline ($10\,mL\,kg^{-1}$); dexamethasone ($10\,mg\,kg^{-1}$, i.p.) followed 30 min later by LPS ($40\,mg\,kg^{-1}$, i.p.).

Statistical analyses

Data are expressed as mean $\pm$ s.e.m. Analysis of the effects of dexamethasone against LPS was undertaken using analysis of variance and a General Linear Model for unbalanced designs (number of mice in each group was unequal). In the studies on the knockout mice the numbers were equal in each group ($n = 8$) and two-way analysis of variance was applied, using LPS and PAR-2 genotype as the factors.

Results

Effect of dexamethasone

Administration of LPS to dexamethasone-treated mice produced the same behavioural effects as those seen in controls (i.e., lethargy).

LPS produced a significant increase in serum ALT concentration (Figure 1A, $P < 0.001$). Dexamethasone did not itself modify ALT concentration significantly, although there was a significant interaction between dexamethasone and LPS, suggesting that the LPS-induced increase in serum ALT concentration was reduced by dexamethasone.

LPS appeared to elevate serum creatinine concentrations (Figure 1B) but analysis of variance showed no statistical significance in this experiment, possibly because of the tendency of dexamethasone to elevate serum creatinine.

Dexamethasone pre-treatment (Figure 1C) had no significant effect on serum glucose ($P = 0.19$) but prevented the LPS-induced hypoglycaemia (analysis of variance; interaction between LPS and dexamethasone, $P = 0.009$).

Serum lipase concentrations were significantly increased by LPS (Figure 1D, $P = 0.01$) but dexamethasone failed to modify significantly this increase (effect of dexamethasone, $P = 0.62$; interaction between dexamethasone and LPS, $P = 0.088$).

LPS had a highly significant effect in elevating lung MPO concentrations (Figure 1E, $P = 0.001$) and there was a significant interaction between dexamethasone and LPS ($P = 0.032$). Scrutiny of the data showed that this interaction suggested a higher increase in lung MPO concentrations in mice receiving LPS and dexamethasone.

At 6 h after injection, analysis of variance (General Linear Model) of the rectal temperature in LPS-treated mice showed that there was a significant elevation, although dexamethasone did not modify this (rectal temperature: saline 35.0 ± 0.4°C, LPS alone 36.1 ± 0.43°C; dexamethasone + saline 34.6 ± 77°C; dexamethasone + LPS 35.7 ± 0.47°C; effect of LPS, $P = 0.038$; effect of dexamethasone, $P = 0.4$; interaction, $P = 0.976$).

Effect of PAR-2 deletion

Because of the shortage of +/+ mice, two of the saline-injected +/+ and one of the LPS-injected +/+ mice were actually original, wild-type C57Bl/6 (from which the colony was derived), so as to give a balanced design. Omission of the data from these mice made no significant difference to

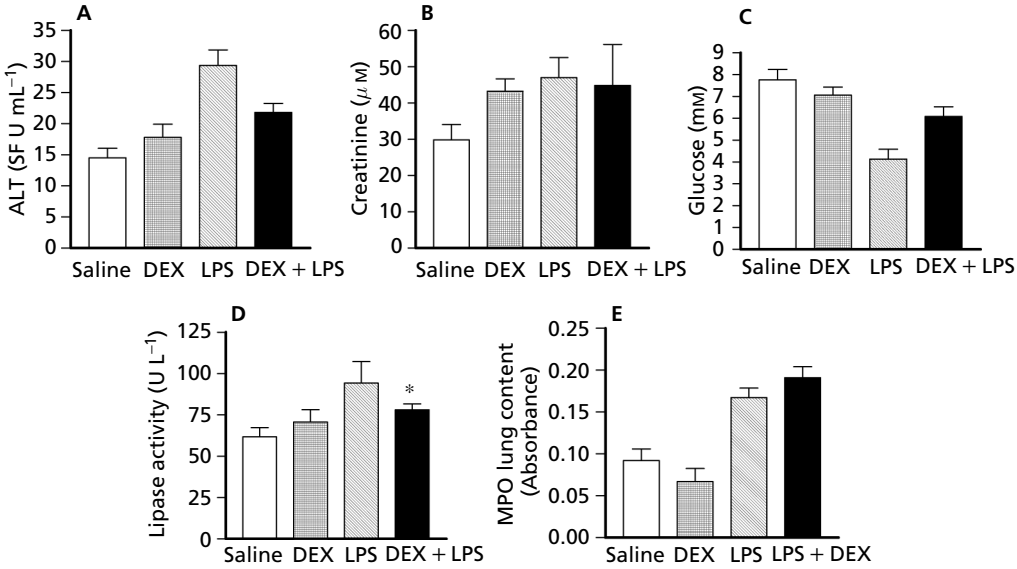

Figure 1 A. Serum concentration of alanine aminotransferase (ALT) in mice receiving saline (n = 8), LPS (n = 6), dexamethasone (DEX; n = 5) or dexamethasone + LPS (n = 8). Data are expressed as mean + s.e.m. Analysis of variance (General Linear Model): effect of LPS, $P < 0.001$; effect of dexamethasone, $P = 0.27$; interaction, $P = 0.009$. B. Serum concentration of creatinine in mice receiving saline (n = 7), LPS (n = 6), dexamethasone (DEX; n = 5) or dexamethasone + LPS (n = 6). Data are expressed as mean + s.e.m. Analysis of variance (General Linear Model): effect of LPS, $P < 0.226$; effect of dexamethasone, $P = 0.46$; interaction, $P = 0.312$. C. Serum concentration of glucose in mice receiving saline (n = 8), LPS (n = 6), dexamethasone (DEX, n = 5) or dexamethasone + LPS (n = 7). Data are expressed as mean + s.e.m. Analysis of variance (General Linear Model): effect of LPS, $P < 0.001$; effect of dexamethasone, $P = 0.19$; interaction, $P = 0.009$. D. Serum concentration of lipase in mice receiving saline (n = 8), LPS (n = 4), dexamethasone (DEX, n = 5) or dexamethasone + LPS (n = 7). Data are expressed as mean + s.e.m. Analysis of variance (General Linear Model): effect of LPS, $P < 0.01$; effect of dexamethasone, $P = 0.62$; interaction, $P = 0.088$. E. Lung content of myeloperoxidase (MPO) in mice receiving saline (n = 8), LPS (n = 6), dexamethasone (DEX, n = 6) or dexamethasone + LPS (n = 6). Data are expressed as mean + s.e.m. Analysis of variance (General Linear Model): effect of LPS, $P < 0.001$; effect of dexamethasone, $P = 0.89$; interaction, $P = 0.032$. LPS (or saline) was administered intraperitoneally ($40\,mg\,kg^{-1}$) 6 h before blood sampling. Dexamethasone (or saline) was administered intraperitoneally ($10\,mg\,kg^{-1}$) 30 min before LPS.

Table 1 Comparison of biochemical values in C57Bl/6 mice with those in +/+ mice from the PAR-2 breeding programme 6 h after the intraperitoneal injection of saline or LPS (40 mg kg^{-1}) and 4 h after induction of anaesthesia with pentobarbital

Mice (treatment)	n	Serum glucose (mM)	Serum creatinine (μM)	Serum ALT (U mL^{-1})	Serum lipase (U L^{-1})	Lung MPO (optical density)
C57Bl/6 (saline)	10–11	7.2 ± 0.4	28.8 ± 2.9	13.1 ± 1.3	63.3 ± 4.0	0.109 ± 0.010
+/+ (saline)	6	8.2 ± 0.8	25.3 ± 1.4	17.6 ± 3.0	61.7 ± 4.2	0.087 ± 0.010
C57Bl/6 (LPS 40 mg kg^{-1})	8–10	4.60 ± 0.84	43.0 ± 3.6	22.0 ± 1.7	88.6 ± 5.5	0.190 ± 0.015
+/+ (LPS 40 mg kg^{-1})	7	4.30 ± 0.83	42.5 ± 3.3	35.5 ± 6.9	90.7 ± 5.1	0.174 ± 0.015

Data are expressed as mean ± s.e.m.

the group means. There were also no significant differences between pentobarbital-anaesthetised +/+ mice (either including or excluding the 3 C57Bl/6 mice) and wild-type C57Bl/6 mice in the serum concentrations of glucose, creatinine, ALT and lipase and in lung MPO at 6 h after intraperitoneal administration of pyrogen-free saline. Moreover, at 6 h after LPS administration, there were no significant differences between pentobarbital-anaesthetised wild-type C57Bl/6 mice and +/+ mice in their serum concentrations of glucose, creatinine and lipase and lung MPO content, although there was a trend to an increased serum ALT concentration (Table 1). Of course, these observations must be viewed with caution, as the wild-type mice were not studied at the same time as the +/+ mice, although the same laboratory conditions were used. In the figures the term wild type denotes the groups of mice consisting predominantly of +/+ mice, together with the small number of C57Bl/6.

The effects of PAR-2 deletion are shown in Figures 2A–E. There were no significant differences between the effects of LPS in the +/+ mice and the −/− mice. The lung MPO activity was significantly lowered in the −/− mice

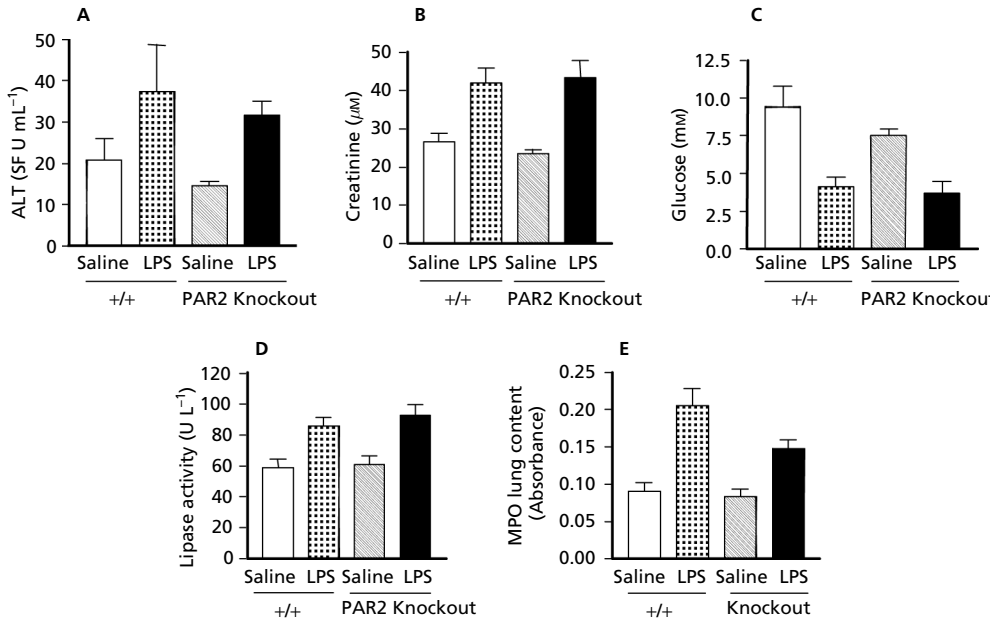

Figure 2 A. Serum concentration of alanine aminotransferase (ALT) in PAR-2 (+/+) or PAR-2 (−/−) (PAR2 knockout) mice receiving saline or LPS (n = 8 for each group). Data are expressed as mean + s.e.m. Analysis of variance: effect of LPS, $P = 0.021$; effect of PAR-2 deletion, $P = 0.36$; interaction, $P = 0.94$. B. Serum concentration of creatinine in wild-type or PAR-2 (−/−) mice receiving saline or LPS (n = 8 for each group). Data are expressed as mean + s.e.m. Analysis of variance: effect of LPS, $P < 0.001$; effect of PAR-2 deletion, $P = 0.59$; interaction, $P = 0.74$. C. Serum concentration of glucose in wild-type or PAR-2 (+/+) mice receiving saline or LPS (n = 8 for each group). Data are expressed as mean + s.e.m. Analysis of variance: effect of LPS, $P < 0.001$; effect of PAR-2 deletion, $P = 0.2$; interaction, $P = 0.47$. D. Serum concentration of lipase in PAR-2 (+/+) or PAR-2 (−/−) mice receiving saline or LPS (n = 8 for each group). Data are expressed as mean + s.e.m. Analysis of variance: effect of LPS, $P < 0.001$; effect of PAR-2 deletion, $P = 0.51$; interaction, $P = 0.73$. E. Lung content of myeloperoxidase (MPO) in PAR-2 (+/+) or PAR-2 (−/−) mice receiving saline or LPS (n = 8 for each group). Data are expressed as mean + s.e.m. Analysis of variance: effect of LPS, $P < 0.001$; effect of PAR-2 deletion, $P = 0.048$; interaction, $P = 0.13$. LPS (or saline) was administered intraperitoneally (40 mg kg^{-1}) 6 h before blood sampling.

Table 2 Rectal temperature 6 h after administration of LPS (40 mg kg^{-1}, i.p.) or saline to mice that are +/+ or −/− for the PAR-2 gene

Treatment (mouse type)	n	Rectal temperature (°C)
Saline (+/+)	7	34.5 ± 0.7
LPS (+/+)	8	35.5 ± 0.6
Saline (−/−)	8	33.4 ± 0.4
LPS (−/−)	8	33.9 ± 0.7

All mice were anaesthetised with pentobarbital (90 mg kg^{-1}, i.p.) at 2 h after injection. Data are expressed as mean ± s.e.m.

(two-way analysis of variance, $P = 0.048$) but the effect of LPS was not significantly different between +/+ and −/− mice (two-way analysis of variance, interaction, $P = 0.132$).

Rectal temperature (Table 2) was significantly lower in −/− mice (analysis of variance comparing all +/+ mice with all −/− mice, $P = 0.038$). However, in this experiment, analysis of variance showed no significant effect of LPS overall (comparing all LPS-treated mice with all saline-treated mice, irrespective of genotype) and no significant interaction between LPS and the knockout genotype ($P = 0.65$).

Discussion

A stable and consistent model of endotoxaemia was produced in the mouse, allowing a 6-h study, while minimising the distress to the mouse by using pentobarbital anaesthesia during the last 4 h of the experiment. In this model LPS produced its well-documented effect of hypoglycaemia (Chang et al 1996; Ruetten & Thiemermann 1997) and an elevation in the serum concentration of ALT, indicating hepatic dysfunction (Ruetten & Thiemermann 1997; Ishii et al 2002), creatinine, indicating renal dysfunction (Ruetten & Thiemermann 1997) and lipase, indicating exocrine pancreatic dysfunction (Ruetten & Thiemermann 1997). Moreover, there was a marked increase in lung content of MPO, indicating pulmonary white cell infiltration, as we had previously shown using electron microscopy in the rat (Fatehi-Hassanabad et al 1996).

The mice showed a hyperthermic response to LPS. The effect of LPS on body temperature in mice is complex and depends on dose and sampling time. Low doses produce an increase in body temperature, whereas higher doses (~2.5 mg kg^{-1}) produce biphasic effects with hyperthermia preceded during the first few hours by hypothermia (Leon et al 1999).

Glucocorticoids are among the very few treatments that have made any impact on experimental endotoxin-induced shock. They have been shown to be effective in some models of LPS-induced shock and organ dysfunction (McKechnie et al 1985; Ruetten & Thiemermann 1997; Han et al 1999; Rocksen et al 2000) and some clinical studies (Briegel et al 1999). In this study, some, but not all of the effects of LPS were attenuated by dexamethasone. Thus, dexamethasone prevented the LPS-induced fall in blood glucose and diminished significantly the LPS-induced elevation in serum ALT concentrations, as reported in the rat (Ruetten & Thiemermann 1997). However, it had no significant effect on indices of renal or exocrine pancreatic dysfunction or on lung MPO activity (a surrogate for neutrophil infiltration and sequestration) or LPS-induced elevation in body temperature. Others have shown dexamethasone to have little effect on the LPS-induced bronchoconstriction and lung neutrophil sequestration in the mouse (Lefort et al 2001). Moreover, dexamethasone failed to inhibit LPS-induced activation of NFκB in-vivo despite being highly effective in this regard in-vitro (Sadikot et al 2001).

Effect of PAR-2 deletion

Responses to LPS were not significantly modified in the PAR-2 −/− mice compared with the +/+ controls. The only detected phenotypic differences between −/− and +/+ mice were the significantly lower rectal temperatures and the significant reduction in lung content of MPO in the −/− mice, although the LPS effects on lung MPO content were not significantly modified. These data must be considered in light of the inclusion in the 16 +/+ mice of 3 wild types (2 receiving saline and one receiving LPS), although, as indicated previously, no differences could be detected between +/+ and wild-type mice and omission of the data from the 3 wild-type mice did not affect the group means. The lower lung MPO levels in −/− mice compared with +/+ mice may agree with the finding that leucocyte rolling was reduced in cremasteric venules of −/− mice early after surgical preparation of the tissue for intra-vital microscopy (Lindner et al 2000). Although the MPO response to LPS was itself not diminished, lower MPO levels may result in reduced lung damage and this may be worth investigating further. The biological significance of these changes remains to be determined. One interpretation of the failure of PAR-2 deletion to modify responses to LPS is that activation of PAR-2 does not play any fundamental role in LPS-induced multi-organ dysfunction. However, this does not mean that the receptor is not activated during endotoxaemia and it is possible that a role may be detected if a longer time period was investigated or a less severe model of LPS-induced organ dysfunction were to be employed. It also remains possible that the role of the PAR-2 may become evident when certain other mediators of the LPS-effects are blocked (e.g. by glucocorticoids, anti-TNFα antibodies or selective inhibitors of iNOS). Indeed, while this manuscript was in preparation, it was reported that, although neither PAR-1 or PAR-2 deletion alone affected LPS-induced IL-6 expression or mortality, survival was significantly prolonged in LPS-treated PAR-2 −/− mice if thrombin-dependent signalling was concomitantly inhibited using hirudin (Pawlinski et al 2003). This suggests that both PAR-2 and thrombin-dependent signalling, probably via PAR-1 or PAR-4, or both, are involved. Experiments using receptor knockout are, of course, subject to the fact that other

systems may be up-regulated during the development of the animal to compensate for the missing receptor. Further elucidation of the role of PAR-2 in LPS-induced shock requires the development of selective antagonists.

Conclusions

These data show, for the first time, that mice lacking the PAR-2 receptor do not differ from genetically equivalent mice that are intact for this receptor in their responses to LPS in producing multi-organ dysfunction, supporting very recent work showing that LPS-induced mortality or IL-6 expression was not modified by PAR-2 deletion alone.

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