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Resistance to endotoxic shock as a consequence of defective NF-κB activation in poly (ADP-ribose) polymerase-1 deficient mice

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Poly (ADP-ribose) polymerase-1 is a nuclear DNA-binding protein that participates in the DNA base excision repair pathway in response to genotoxic stress in mammalian cells. Here we show that *PARP-1*-deficient cells are defective in NF-κB-dependent transcription activation, but not in its nuclear translocation, in response to TNF-α. Treating mice with lipopolysaccharide (LPS) resulted in the rapid activation of NF-κB in macrophages from *PARP-1*$^{+/+}$ but not from PARP-1$^{-/-}$ mice. *PARP-1*-deficient mice were extremely resistant to LPS-induced endotoxic shock. The molecular basis for this resistance relies on an almost complete abrogation of NF-κB-dependent accumulation of TNF-α in the serum and a down-regulation of inducible nitric oxide synthase (iNOS), leading to decreased NO synthesis, which is the main source of free radical generation during inflammation. These results demonstrate a functional association *in vivo* between PARP-1 and NF-κB, with consequences for the transcriptional activation of NF-κB and a systemic inflammatory process.

Keywords: NF-κB/nitric oxide/poly (ADP-ribose) polymerase/septic shock/TNF-α

Introduction

PARP-1 is a nuclear zinc-finger DNA-binding protein that detects specifically DNA-strand breaks generated by different genotoxic agents (de Murcia and Ménissier-de Murcia, 1994). PARP-1 is associated *in vivo* with XRCC1, a DNA repair protein involved, together with DNA pol β and DNA ligase III, in base excision repair (Masson *et al*., 1998) of DNA. Treatment of *PARP-1*$^{-/-}$ mice with either alkylating agents or γ-irradiation reveals an extreme sensitivity and a high genomic instability to both agents. Following whole body γ-irradiation, mutant mice died rapidly from acute radiation toxicity of the small intestine (Ménissier-de Murcia, 1997). Cells derived from these mice display retarded kinetics of DNA ends rejoining following damage with an alkylating agent (Trucco *et al*., 1998), indicating that PARP-1 is involved in base excision repair, and preferentially in the long patch pathway, probably by recruiting DNA repair enzymes to the vicinity of a DNA lesion (Dantzer *et al*., 1999). Genetic deletion of *PARP-1* attenuates tissue injury after transient cerebral ischemia (Eliasson *et al*., 1997), streptozotocin-induced diabetes (Burkart *et al*., 1999; Masutani *et al*., 1999), and pharmacological inhibition of PARP-1 improves the adverse clinical effects in different pathologies associated with inflammation (Szabo and Dawson, 1998).

Septic shock is the most common cause of death in intensive care units and it is usually the result of a systemic Gram-negative bacterial infection resulting in hypotension and failure of a number of organ systems, in particular the liver, kidney and heart (Parrillo, 1993). The bacterial membrane component lipopolysaccharide (LPS), when injected into animals, causes a shock-like state leading to death. The mechanism by which LPS induces endotoxic shock is related to its ability to activate the NF-κB/Rel family of transcription factors, enabling the expression of several critical genes involved in the pathogenesis of septic shock: TNF-α, interleukins (IL-1β, IL-2, IL-6 and IL-8), adhesion molecules (I-CAM-1 and E-selectin), cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) (Ghosh *et al*., 1998).

One mechanism proposed for the toxicity of free radicals produced during inflammation includes the induction of DNA single-strand breaks and over-activation of PARP-1, resulting in a massive depletion of cellular energy and in necrotic cell death (Szabo *et al*., 1996). In the present study we describe an alternative and unexpected mechanism by which PARP-1 is functionally associated with the transcription factor NF-κB. Through this property, in the absence of PARP-1, NF-κB-dependent transcription is impaired, and the *in vivo* release of inflammatory mediators is down-regulated during endotoxic shock. As a consequence. *PARP-1*-deficient mice are extremely resistant to lethality induced by LPS. NF-κB activation is upstream of the synthesis of acute phase inflammatory mediators, and could explain the decreased incidence of inflammatory problems in *PARP-1*-null mice. New anti-inflammatory and anti-sepsis therapy might be designed by targeting on this interaction between PARP-1 and NF-κB.

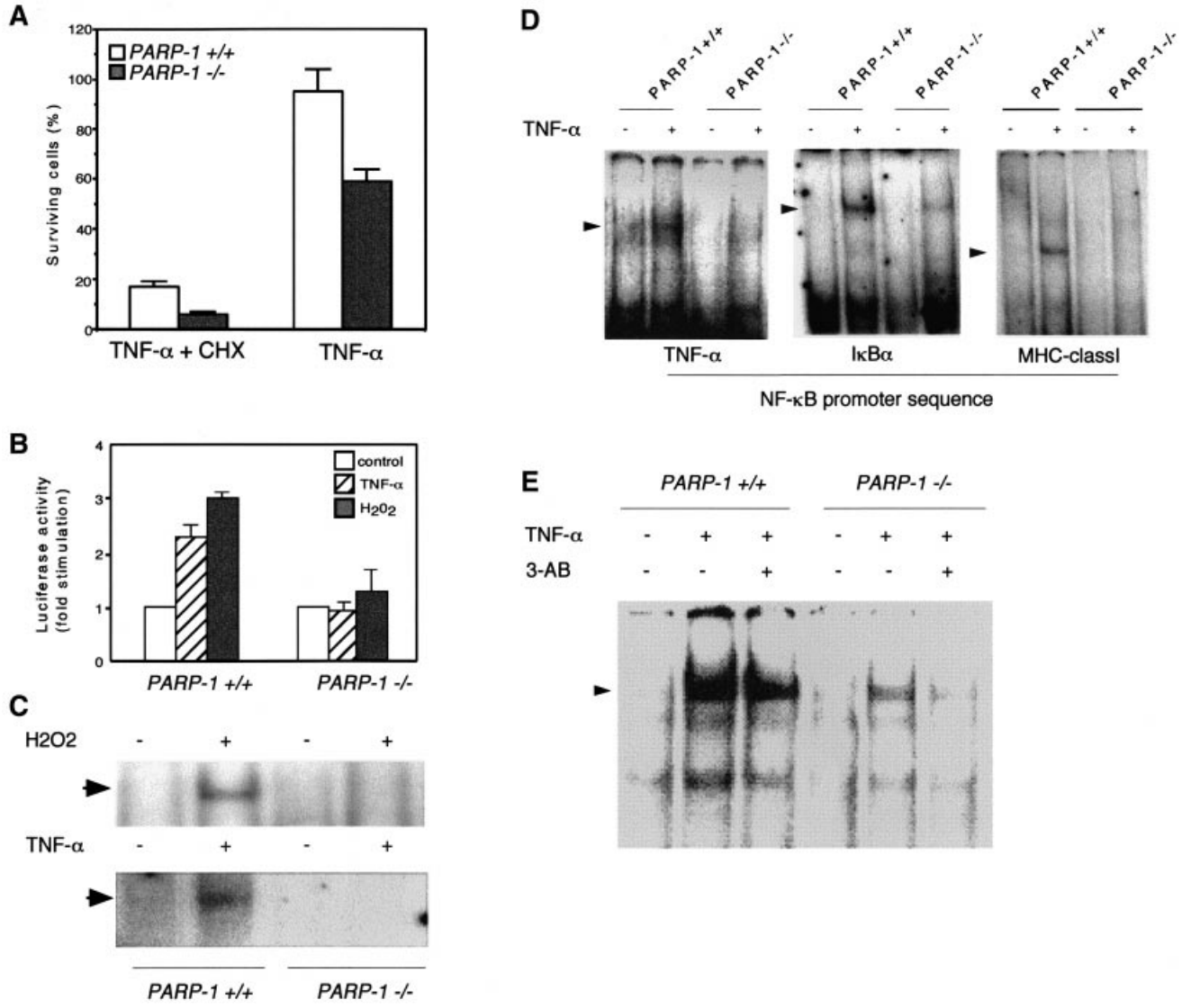

Fig. 1. Defective NF-κB activation in *PARP-1*-deficient cells. (**A**) Increased cytotoxic effect of TNF-α in *PARP-1*$^{-/-}$ cells. Immortalized 3T3 cells were treated with mouse recombinant TNF-α (50 ng/ml) alone or in combination with cycloheximide (CHX) (1 μg/ml) and surviving cells were determined at 48 h by trypan blue dye exclusion. The results represent the average of three independent experiments. (**B**) NF-κB-dependent transcriptional activation was determined by transient transfection of a luciferase reporter plasmid under the control of a 3×κB promoter element. After 48 h, cell were treated with TNF-α (10 ng/ml) for 6 h, or 1 mM H_2O_2 for 10 min and cell lysates were prepared after 6 h. Results are normalized for β-gal expression. In (**C**), NF-κB activation was analyzed by band shift assay, as detailed in the Materials and methods, and the time of treatment was 30 min with the same dose as above. (**D**) Band-shift analysis of NF-κB activation using three different κB responsive elements in the TNF-α, IκBα and MHC class I genes promoters. 3T3 cells were stimulated for 30 min with 50 ng/ml of TNF-α and EMSA was performed as detailed in Materials and methods. (**E**) Effect of the PARP-1 inhibitor 3-aminobenzamide (3-AB) on NF-κB activation. 3T3 cells of both genotypes were preincubated with 2 mM 3-AB for 3 h before TNF-α treatment.

Results

PARP-1-deficient cells are more sensitive to cytotoxicity induced by TNF-α and are defective in NF-κB activation

We have previously reported that *PARP-1*-deficient cells are more sensitive than wild-type (wt) cells to apoptosis induced by DNA-damaging agents that activate the base excision repair pathway (Ménissier-de Murcia, 1997; Oliver *et al*., 1998). Since activation of NF-κB has been shown to protect against the cytotoxicity of some chemotherapeutic drugs and TNF-α, we have now studied the effect of TNF-α (Van Antwerp *et al*., 1998) on the viability of *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ immortalized mouse embryonic fibroblasts (3T3) (Figure 1A). Treating 3T3 cells with TNF-α + cycloheximide resulted in a dramatic loss of cell viability for the two genotypes; TNF-α alone induced a considerable cytotoxic effect in *PARP-1*-deficient cells, while *PARP-1* wt cells were mostly unaffected (Figure 1A). This increased cytotoxic effect of TNF-α could be related to impaired NF-κB activation in *PARP-1*$^{-/-}$ cells. Thus, the transcriptional status of NF-κB was examined in transiently transfected 3T3 cells of both genotypes stimulated with TNF-α (for 3 h) or H_2O_2 (30 min) using a luciferase reporter plasmid under the control of a 3×κB consensus site from the HIV enhancer (Bachelerie *et al*., 1991). Stimulation of 3T3 cells with TNF-α or H_2O_2 increased luciferase activity in transfected *PARP-1*$^{+/+}$ cells, but not in *PARP-1*$^{-/-}$ cells, suggesting that NF-κB transcriptional activation was impaired in

PARP-1$^{-/-}$ cells (Figure 1B). Then, the activation of NF-κB in *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ 3T3 cell lysates was analysed by band shift assay, using the NF-κB responsive element in the iNOS promoter (Xie *et al*., 1994). Only in *PARP-1*$^{+/+}$ cells was a band-shift observed after treatment with these agents (Figure 1C). The specificity of the shifted band was tested using a 50-fold excess of non-labelled DNA (not shown).

Although classic NF-κB is a heterodimer of a 50 kDa subunit (NF-κB1 or p50) and a 65 kDa subunit (Rel A or p65), other subunits might be constituents of the transcription factor (Ghosh *et al*., 1998), and a number of κB elements have been described for which the different NF-κB heterodimers present diverse binding affinities, allowing a fine tuning of the transcription of the large variety of genes induced by NF-κB. To substantiate further our observations, we performed a gel-retardation assay with three different NF-κB responsive elements (for TNF-α, IκBα and MHC class I gene promoters); in all three cases a band-shift was observed in extracts from *PARP-1*$^{+/+}$ cells treated with TNF-α, but a substantial decrease was observed in extracts from *PARP-1*-mutant cells (Figure 1D) stimulated with TNF-α. For the IκBα promoter sequence, a significant decrease in the shifted band has always been observed but never a complete inactivation, suggesting a differential effect of PARP-1 on the transcription of these genes.

A decreased capacity of a murine macrophage cell line to activate NF-κB following treatment with the PARP-1 inhibitor 3-aminobenzamide (3-AB) has been reported (Le Page *et al*., 1998). To test whether the observed decrease in NF-κB activation was the result of the lack of PARP-1 activity or due to the absence of the protein itself, we have studied the effect of the PARP-1 inhibitor 3-AB (2 mM) (Figure 1E). We have found a small decrease in NF-κB-DNA binding activity using the iNOS promoter sequence in nuclear extracts from 3-AB treated cells, which does not explain the large defect in the activation of NF-κB in *PARP-1*$^{-/-}$ cells. Although moderate, the effect of 3-AB was also detected in *PARP-1*$^{-/-}$ cells, possibly due to the presence of another DNA-dependent PARP activity in *PARP-1*$^{-/-}$ cells (Amé *et al*., 1999; Berghammer *et al*., 1999). This result suggests that the protein itself, rather than its activity, potentiates NF-κB transcriptional activation, probably through the interaction of PARP-1 with the transcription complex, as has been described for other transcription factors (Nie *et al*., 1998; Oei *et al*., 1998; Butler and Ordahl, 1999; Plaza *et al*., 1999).

IκBα proteolysis and NF-κB/p65 nuclear translocation are not affected in PARP-1$^{-/-}$ cells

NF-κB proteins are constitutively present in the cell, but they are retained in the cytoplasm associated with inhibitory proteins known as IκBs, the degradation of which represents the key regulatory event of NF-κB-dependent transcription. Activated NF-κB complexes are translocated to the nucleus and this requires phosphorylation and degradation of the IκB proteins (Finco and Baldwin, 1995). Therefore, we have determined the half-life of IκBα, the most potent κB inhibitory protein, in 3T3 cells of both genotypes, following treatment with TNF-α. In agreement with previous results (Krappmann *et al*., 1996), IκBα was rapidly degraded in response to TNF-α treatment (Figure 2A, bottom panel) and resynthesized thereafter. Similarly, accumulation of IκBα mRNA levels after TNF-α treatment were unaffected in *PARP-1*-deficient cells (results not shown). By indirect immunofluorescence (Figure 2B), we confirmed that the NF-κB/p65 was translocated to the nucleus in *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ cells, suggesting that this step was not affected in the *PARP-1*-null cells. p65 and p50 subunits of NF-κB were similarly expressed in both genotypes as determined by Western blotting using either cultured cells or different tissue extracts (not shown). Altogether, the results presented so far indicate that early steps between membrane- or receptor-linked events, and NF-κB translocation to the nucleus are not affected in *PARP-1*$^{-/-}$ cells. All these results suggest that PARP-1 meets NF-κB after the translocation of the κB heterodimer to the nucleus, and the most likely step is the formation of the transcription complex, altering the DNA binding affinity of NF-κB.

PARP-1-deficient mice are resistant to endotoxic shock induced by LPS

Signalling through NF-κB integrates the transcriptional activation of a number of genes whose products regulate critical processes of the inflammatory response. We have studied in the animal the consequences of having a decreased NF-κB response in a *PARP-1*-knockout context. Intra-peritoneal injection of a high dose of LPS in mice induces lethal endotoxic shock. *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ mice of 6–8 weeks of age were injected with 40 mg/kg body weight of LPS. Both wild-type and *PARP-1*-deficient mice equally showed a series of responses such as fever, diarrhoea and lethargy. However, while only 10% of the wild-type mice survived 1 day after receiving LPS, 90% of *PARP-1*-deficient mice recovered and survived for many days (surviving mice were sacrificed 3 weeks after LPS treatment) (Figure 3A). In *PARP-1*$^{-/-}$ mice, a loss of weight of ~10% was noted during the first 48 h following LPS injection, from which they recovered afterwards. Furthermore, the level of NF-κB activation following LPS injection was determined using nuclear extracts of peritoneal macrophages obtained from control or LPS-treated mice (Figure 3B). A deficient activation in macrophages from *PARP-1*-mutant mice injected with LPS was found again, as we showed above for cultured *PARP-1*$^{-/-}$ cells.

LPS-induced synthesis of inflammatory mediators

Treatment of normal mice with LPS results in the increased production of a number of cytokines and inflammatory mediators (Laubach *et al*., 1995), including the upregulation of iNOS protein, by activating NF-κB. Macrophages are one of the main sources of iNOS during septic shock. We determined the level of the iNOS protein following LPS treatment. Western blots showed an increased expression of iNOS in macrophages withdrawn from *PARP-1*$^{+/+}$ mice challenged with LPS (Figure 3C), but not from *PARP-1*-deficient mice injected with LPS. Experiments using primary cultured macrophages, showed that LPS-induced NO release (determined as nitrite accumulation in the culture medium) was also diminished in cells from *PARP-1*-deficient mice (Figure 3D) compared with cells from *PARP-1*$^{+/+}$ mice.

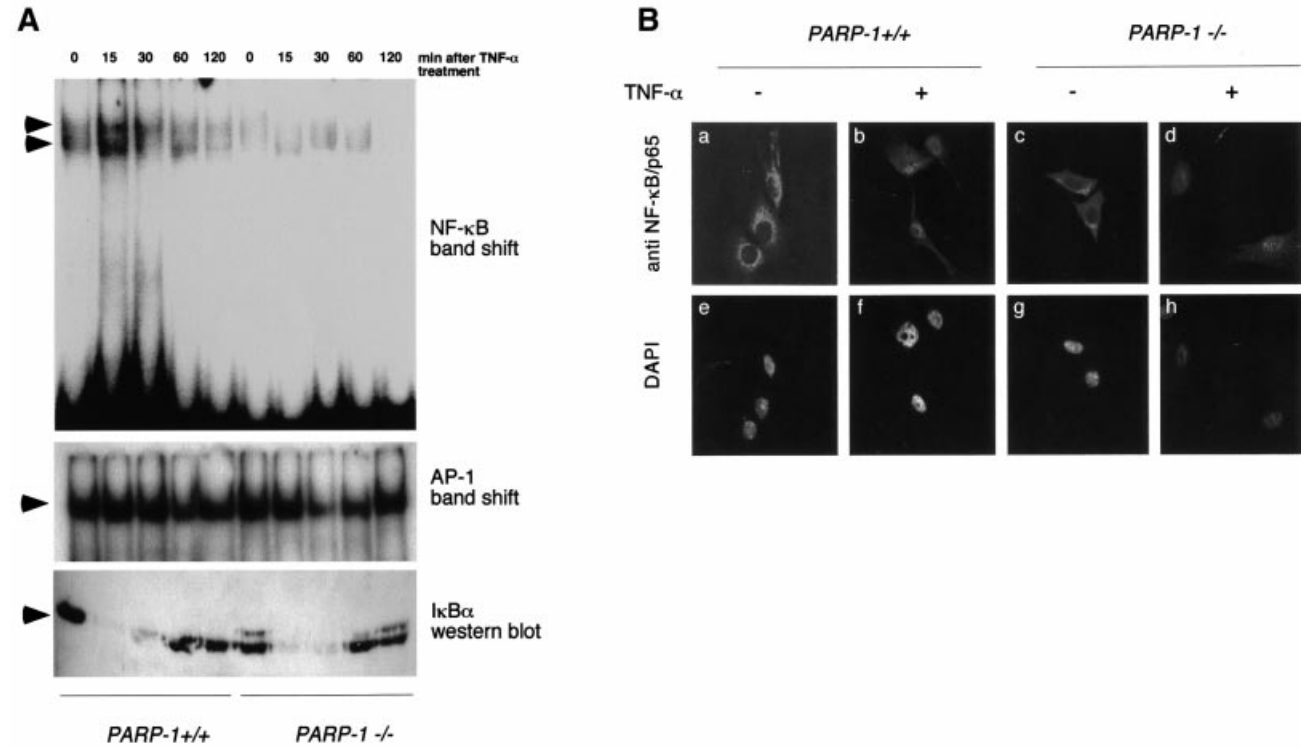

Fig. 2. Kinetics of IκBα degradation and nuclear translocation of p65 in *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ 3T3 cells. (**A**) The band-shift experiment was performed by incubating nuclear extracts from TNF-α-treated (50 ng/ml of murine recombinant TNF-α, for the times indicated) or untreated cells with the ^{32}P-labelled iNOS promoter responsive sequence, as described in the Materials and methods. An AP-1 consensus sequence was used as control. Cytosolic extracts of the same cells were used for Western blot analysis of IκBα protein. (**B**) Indirect immunofluorescence of NF-κB/p65 was performed after treatment of the cells with TNF-α (10 ng/ml) for 30 min. (**C**) Supershift assay of the complex composition in the κB site of the iNOS promoter.

TNF-α is a key mediator in the pathology of sepsis, and plays a central role in initiating and regulating the cytokine cascade (Gutierrez-Ramos and Bluethmann, 1997). The serum levels of TNF-α increased significantly in *PARP-1*$^{+/+}$ mice following LPS administration, but in *PARP-1*$^{-/-}$ this increase was significantly reduced ($p < 0.02$) (Figure 4A). The increase in serum levels of IFN-γ (which is also a key mediator of septic shock) after LPS injection was significantly reduced ($p < 0.05$) in *PARP-1*$^{-/-}$ mice (Figure 4B). In contrast, levels of IL6 were identically elevated in both genotypes (Figure 4C). These results are in agreement with a recent report suggesting a role for IL-6 in the development of fever and cachexia in endotoxic shock (as described above for *PARP-1*$^{-/-}$ mice), whereas TNF-α is responsible for the initial hypothermia and lethality of sepsis (Leon *et al*., 1998). Moreover, the fact that IL-6 levels were identically elevated in both mice strains after LPS challenge reveals that mutant mice are not completely unresponsive to the endotoxin, and suggests that LPS receptor is functional in *PARP-1*-deficient mice.

Vascular reactivity during endotoxic shock

In human septic shock and in animals injected with endotoxin, vessels become hypo-responsive to vasoconstrictor agents, and this peripheral vascular failure is attributed to massive NO production by iNOS. To test directly the contractile responses during septic shock, and the NO contribution to the regulation of vascular reactivity in mice challenged with LPS, aortic rings from both *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ mice were obtained to measure isometric force. The contractile response to the prostaglandin analogue U46619 was not significantly different *ex vivo* in the two mice strains (data not shown). Also, treatment of mice from both genotypes with LPS did not modify the contractile response of the vessels to U46619 (Figure 5A). A well known selective inhibitor of the inducible NO-synthase, called SMUT (S-methylisothiourea) (Szabo *et al*., 1994), was used to investigate the participation of NO in the regulation of contraction in vessels from LPS-treated mice. SMUT had no effect on the response to U46619 in control arteries from both strains (not shown). However in the presence of SMUT, the contractile response of aortic rings from *PARP-1*$^{+/+}$ mice treated with LPS was significantly enhanced compared with that of rings from *PARP-1*$^{-/-}$ mice treated with LPS (Figure 5B). Comparison between the curves in Figure 5A and B shows that SMUT significantly increased the contractile effect of U46619 in arteries from *PARP-1*$^{+/+}$ ($p < 0.01$) but not in those from *PARP-1*$^{-/-}$ LPS-treated mice. Furthermore, blockade of the main effector of NO, guanylyl cyclase, with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Garthwaite *et al*., 1995), enhanced contractile response to U46619 in aortic rings from both *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ mice treated or not with LPS (Figure 5C). After LPS, the potentiating effect of ODQ was greater in arteries from *PARP-1* $^{+/+}$ than in those from *PARP-1*$^{-/-}$ mice. Interestingly, the effect of ODQ was not different in control arteries from both strains, suggesting that endogenous endothelium-derived NO

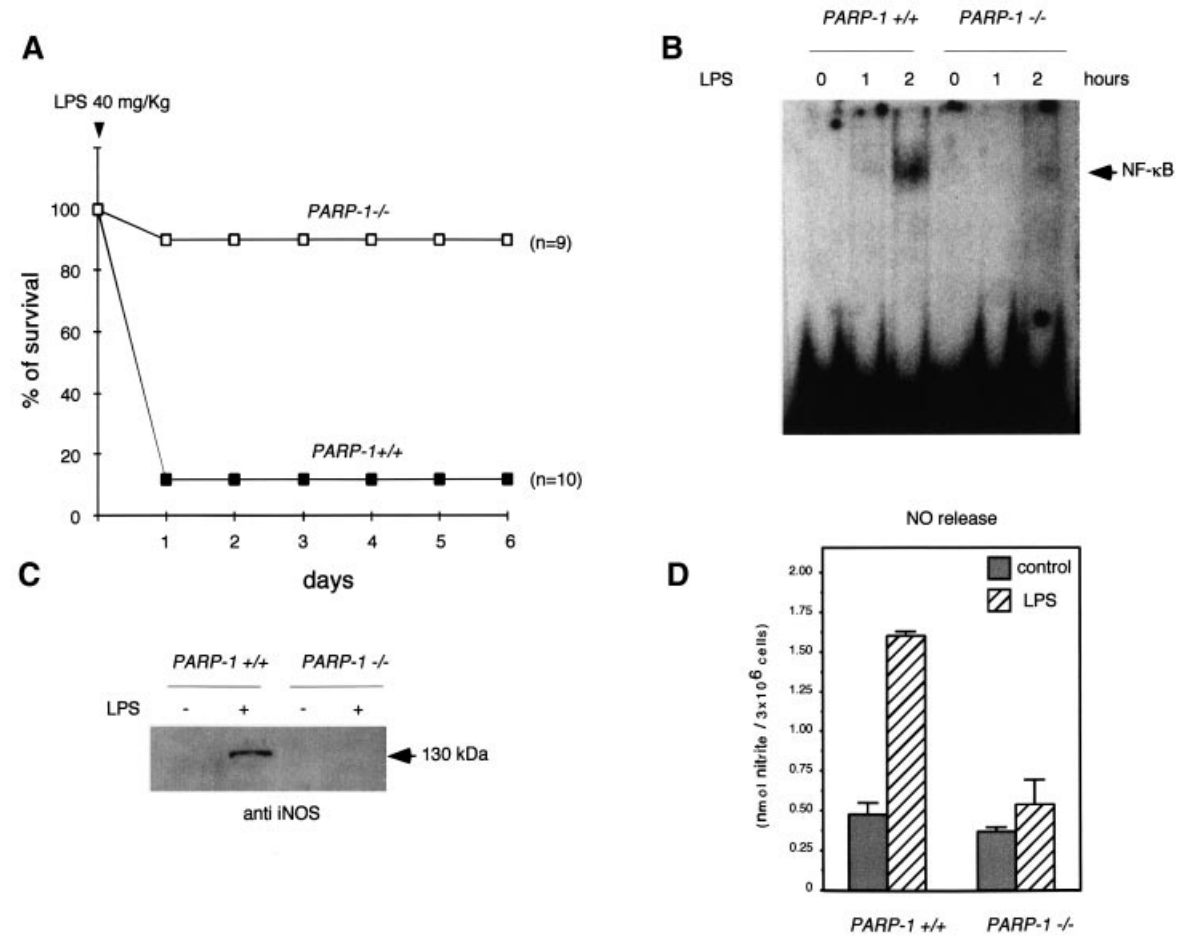

Fig. 3. (**A**) Mice survival after i.p. injection of LPS (40 mg/kg of body weight). The number of mice is given in the plot. (**B**) NF-κB activation in peritoneal macrophages from LPS (40 mg/kg)-challenged mice. Band shift was performed as described in the Materials and methods, using the iNOS promoter-responsive element. (**C**) Eighteen hours after LPS treatment, iNOS expression was determined, by Western blotting, in peritoneal macrophages from wild-type and *PARP-1*-deficient mice. (**D**) Nitrite release in primary cultured murine macrophages from *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ mice control (open columns) or treated with 1 μg/ml LPS (hatched columns) for 24 h.

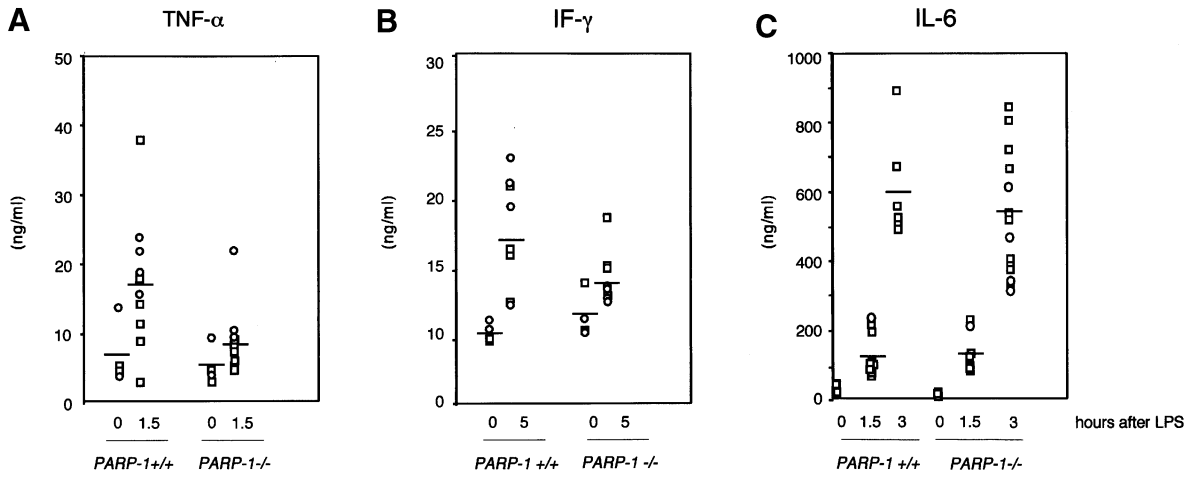

Fig. 4. Cytokines released in LPS-challenged mice. Serum levels of TNF-α (**A**), IF-γ (**B**) and IL-6 (**C**) after i.p. injection were quantitated by ELISA. (□) male; (○) female. The number of observations ranged from four (non-treated animals) to 12. $p < 0.02$ for TNF-α and $p < 0.05$ for IF-γ increases in *PARP-1*$^{-/-}$ mice compared with *PARP-1*$^{+/+}$ mice.

activity was unchanged in *PARP-1*-deficient mice. These results indicate that only in *PARP-1*$^{+/+}$ mice does iNOS-produced NO contribute to the regulation of vascular tone after LPS treatment. To confirm this finding further, we determined the level of iNOS in lysates of the mesenteric artery from LPS-treated or control mice of both genotypes.

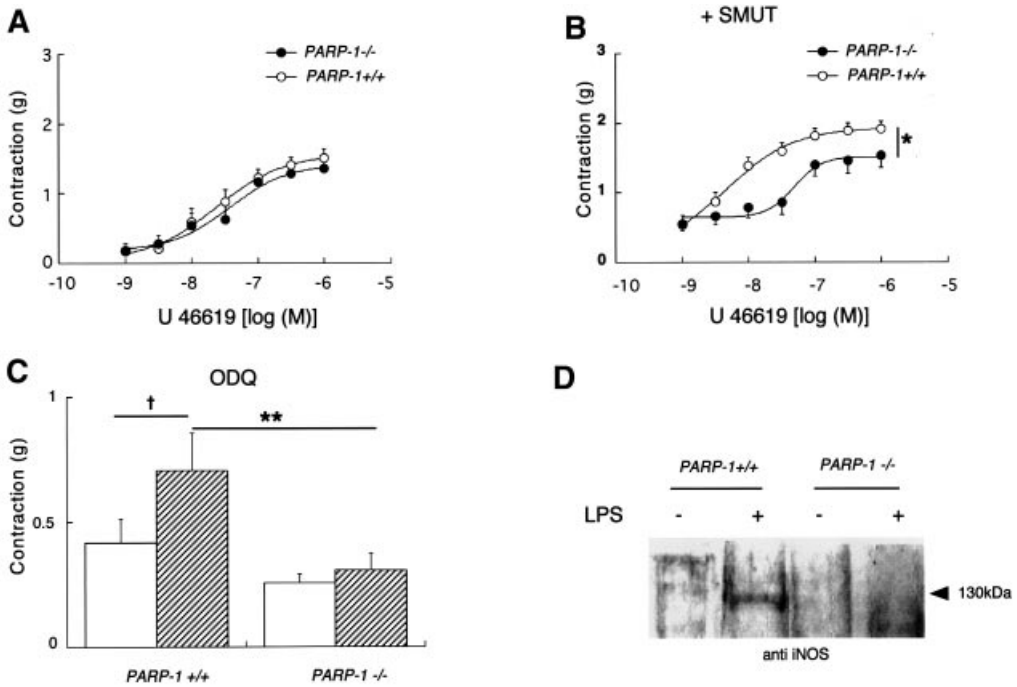

Fig. 5. Contribution of NO to the contractile response of aortae after treatment with LPS. Segments of aortic rings with functional endothelium were mounted 8 h after treating the mice with saline or LPS (40 mg/kg) in a myograph filled with physiological salt solution kept at 37°C and continuously gassed with a mixture of 95% O_2:5% CO_2 (pH 7.4), and mechanical activity was recorded isometrically. Concentration-response curves were constructed by cumulative application of the thromboxane A_2 agonist (9,11-dideoxy-11α, 9-epoxymethano-prostaglandine F_2) U46619 in aortae from LPS-treated mice (n = 5–8) (**A**). In parallel experiments, concentration–response curves to the agonist were performed after 20 min pre-incubation of the arteries with the NO synthase inhibitor, SMUT (100 μM) in aortae from LPS-treated mice (**B**). In aortic rings pre-contracted with 1 mM U46619, the guanylyl cyclase inhibitor ODQ (1 μM) produced a further increase in tension both in control (open columns) or LPS-treated (hatched columns) mice from both groups (**C**). $*p < 0.05$, $**p < 0.01$, significant difference between *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$, and $^{\dagger}p < 0.01$, significant difference between control and LPS-treated *PARP-1*$^{+/+}$ using analysis of variance. (**D**) Western blot of iNOS in lysates from mesenteric arteries of control or LPS-treated mice.

After 8 h of LPS treatment, there was a sharp increase in iNOS levels in wild-type animals, while in mutant mice there was a complete absence of the protein (Figure 5D).

Discussion

Functional association between PARP-1 and NF-κB

The data presented in this study suggest that there is a defective NF-κB activation in the absence of PARP-1 that is not related to an impairment of the signal transduction pathway. The results in Figure 2A show that the rate of re-accumulation of the regulatory protein IκBα (whose gene is under the transcriptional control of NF-κB), is normal in *PARP-1*-deficient cells. This result suggests that the association of PARP-1 with NF-κB modulates the expression of certain genes under the control of NF-κB, but not all of them. Partial inhibition of NF-κB DNA binding alone seems not to be sufficient for down-regulation of the *IκBα* gene.

An earlier report suggested a decreased ability to transactivate through NF-κB following inhibition of PARP-1 with 3-AB (Le Page *et al*., 1998), with consequences for iNOS gene transcription. In this respect, an increasing number of transcription factors or transcription coactivators have been reported to be associated with PARP-1, including AP-2 (Kannan, 1999), oct-1 (Nie *et al*., 1998), YY1 (Oei *et al*., 1997) and TEF-1 (Butler and Ordahl, 1999).

The molecular dissection of the interaction between PARP-1 and the transcriptional machinery of NF-κB still remains to be elucidated. Efforts to show a physical association between PARP-1 and p65 or p50 subunit by immunoprecipitation experiments were not conclusive (data not shown). One possibility might be that PARP-1 and NF-κB contact through a third protein involved in the architectural regulation of transcription, the high mobility group-I protein [HMG-I(Y)], which is a well known coactivator of NF-κB-dependent transcription (Thanos and Maniatis, 1995; Perrella *et al*., 1999) and a substrate for PARP-1 (Tanuma and Johnson, 1983). This is also an attractive possibility since PARP-1 has a high binding affinity for DNA bends (Gradwohl *et al*., 1987; Sastry and Kun, 1990) such as those induced in the NF-κB enhanceosome by HMG-I(Y) (Thanos and Maniatis, 1995).

While this publication was under review, a study by Hassa and Hottiger (1999) has also found a defective NF-κB activation in PARP-1$^{-/-}$ cells.

Resistance of PARP-1$^{-/-}$ mice to endotoxic shock

The response to endotoxin-induced septic shock has been tested in a number of genetically modified mice, including those for iNOS and TNF-α. The *iNOS*$^{-/-}$ mice were not resistant to LPS-induced death (Laubach *et al*., 1995; MacMicking *et al*., 1995), while *TNF*-α-deficient mice were resistant to the lethality of low doses of LPS (administered with D-gal), but not to high doses of endotoxin (Marino *et al*., 1997). In both cases, the persistence of LPS sensitivity could be explained by the existence of NO- and TNF-independent pathways to LPS-

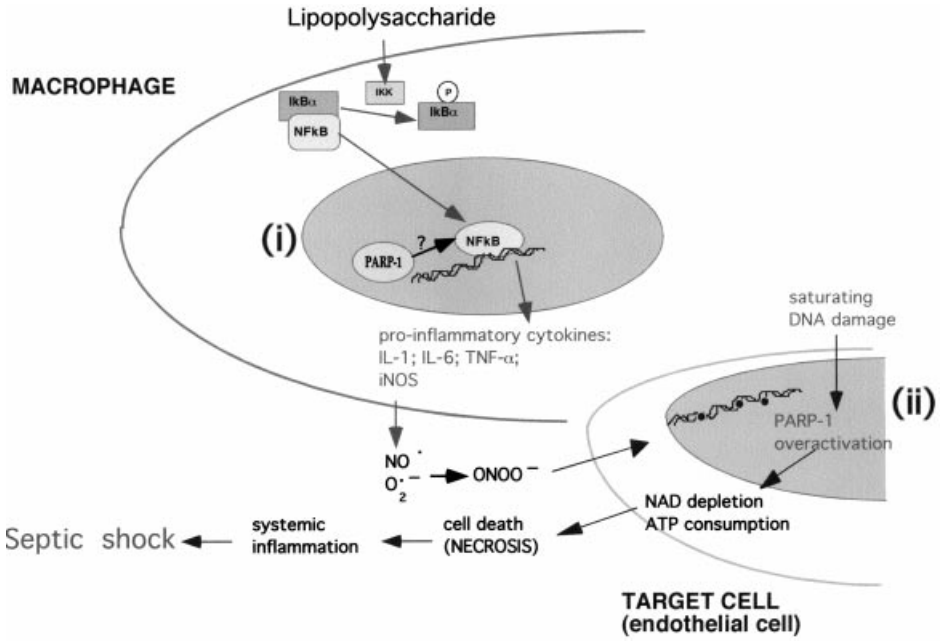

Fig. 6. PARP-1 is involved in the inflammatory response at two different levels: (i) activation of NF-κB and synthesis of proinflammatory factors and (ii) increasing the sensitivity of cells (particularly endothelial cells) to oxygen radicals produced during inflammation with consequences on DNA damage, energy depletion and necrosis (see Discussion). IKK, IκB kinase.

induced death. The resistance of *PARP-1*-deficient mice to septic shock is, however, more dramatic than that reported for iNOS and TNF-α knockout mice, and is similar to that found for interleukin-1β converting enzyme (ICE)-deficient mice (Li *et al*., 1995). Current hypotheses for the pathogenesis of septic shock are that microbial products such as LPS induce a massive production and release of TNF-α by macrophages, which in turn induce a cascade of cytokine production. TNF-α has profound effects on vascular endothelial cells, leading to cell adhesion, vascular leakage and shock (Gutierrez-Ramos and Bluethmann, 1997). As a consequence, neutralization of TNF-α prevents lethality in animal models of sepsis. The reduced synthesis of TNF-α (the gene for which is under the transcriptional control of NF-κB) in *PARP-1*-mutant mice might be relevant to explain the resistance to LPS-induced lethality in these mice.

Role of PARP-1 in the inflammatory response

Studies from several laboratories have noted the participation of PARP-1 in the inflammatory response, and different mechanisms have been proposed to explain that the inactivation of PARP-1 (either pharmacologically or using genetically engineered mice lacking PARP-1), improve in the animal the outcome of a variety of pathophysiological conditions associated with an exacerbated tissue or systemic inflammation (Szabo and Dawson, 1998).

The most extended model implicates PARP-1 in the following pathway: after an inflammatory stress (as LPS treatment induces in the model of endotoxic shock) or during reperfusion after cerebral ischemia, different cells, including macrophages and endothelial cells, activate a massive synthesis of NO, which is in turn converted into a cytotoxic derivative, peroxynitrite. Rapid DNA single-stranded breaks are induced by peroxynitrite, leading to over-activation of PARP-1 and depletion of cellular energy resulting in mitochondrial free radical generation and cell necrosis (Figure 6, ii) (Szabo *et al*., 1996, 1997).

In the present study, we have found a functional association between PARP-1 and NF-κB, a key regulatory molecule involved in multiple pathologies (Barnes and Karin, 1997; Kitamura *et al*., 1997), and particularly in inflammation (Wong *et al*., 1998). Through this association, PARP-1 may regulate NF-κB-dependent transcription, and the synthesis of inflammatory mediators (Figure 6, i). Thus, PARP-1 appears to promote inflammation at two levels, and through its effects on NF-κB and by mediating the cytotoxicity of NO-derivatives, one or both of these mechanisms might also explain the resistance of *PARP-1*$^{-/-}$ mice to brain ischemia, where synthesis of NO by different isoforms of NOS, TNF-α upregulation and NF-κB activation play a crucial role (Iadecola *et al*., 1995; Barone *et al*., 1997; Schneider *et al*., 1999).

Three recent studies carried out in *PARP-1*-deficient mice generated in two different laboratories have also shown an increased resistance of *PARP-1*-mutant mice to streptozotocin-induced diabetes (Burkart *et al*., 1999; Masutani *et al*., 1999; Pieper *et al*., 1999). Interestingly, as an autoimmune disease, the pathogenesis of diabetes is also related to inflammatory damage of pancreatic islet β-cells. Consequently, we propose that a common mechanism involving NF-κB activation is a critical molecular event involved in the pathogenesis of different diseases such as septic shock, brain ischemia, type-1 diabetes or arthritis, from which *PARP-1*-deficient mice are largely protected.

In conclusion, the data presented here, as a whole, demonstrate that PARP-1 is involved in the regulation of the NF-κB signalling pathway leading to synthesis of inflammatory mediators, and in the development of LPS-induced endotoxic shock. This finding might help to design new therapeutic strategies for treatment of septic shock,

based on the combined pharmacological inhibition of NF-κB and PARP-1 (Szabo and Dawson, 1998), allowing the effective down-regulation of pro-inflammatory cytokines by turning off NF-κB-dependent transcription (Liu *et al*., 1997), and reducing cell injury through inhibition of PARP-1. In any case, all approaches that aim to eliminate PARP-1 or PARP-1 activity from the cell or the organism should take into account that since this enzyme is involved in genomic surveillance, its long-term inhibition might lead to the accumulation of DNA damage, mutations and oncogenic transformation.

Materials and methods

Cell culture and transient transfection

Immortalized mouse embryonic fibroblasts (3T3) from either *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ mice were cultured at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 0.5% gentamicin (Sigma), 4.5% glucose. Cells were transiently co-transfected by electroporation with 3 μg of *LacZ*-expressing pCHK reporter vector (as control of transfection) and 15 μg of the NF-κB/luciferase plasmid. Thirty-six hours after transfection, cells were stimulated with murine TNF-α (50 ng/ml) for 3 h or H_2O_2 (1 mM) 30 min, and luciferase activity was determined according to the manufacturer's instructions (Promega). Primary macrophages were obtained from the peritoneal exudate by fluxing with sterile phosphate-buffered saline (PBS), and cultured at 10^6/ml density in DMEM without phenol red, 10% decomplemented FBS and 0.5% gentamicin. The medium was changed 4 h later and floating cells were removed.

Endotoxic shock

Mice C57BL/6 were injected i.p. with LPS from *Escherichia coli* 0111:B4 (Sigma, St Louis, MO) at a dose of 40 mg/kg body weight. The injected mice were monitored for signs of endotoxemia and lethality at least twice a day. The systemic release of cytokines was determined by ELISA for murine TNF-α, IFN-γ and IL-6, 1.5, 3 or 5 h after LPS injection, respectively. Nitrite release in the culture medium of murine macrophages was determined by the Griess reaction as described previously (Szabo *et al*., 1994), following stimulation *in vitro* with 1 μg/ml of LPS for 24 h.

Electrophoretic mobility shift assays (EMSAs)

Nuclear and cytoplasmic extracts were prepared as described previously by Velasco *et al*. (1997). Oligonucleotides were synthesized by Eurogenetech (Belgium). The oligonucleotide sequences corresponding to the consensus NF-κB binding sites of different promoters were used. These sequences were the following:

INOS promoter: 5′-TGCTAGGGGGATTTTCCCTCTTCTTGT-3′ (Xie *et al*., 1994),
IκBα promoter: 5′-GATCTTGGAAATTCCCCGA-3′ (Le Bail *et al*., 1993),
MHC class I promoter: 5′-TGGGCTGGGGATTCCCCATCTCAGCT-3′, (Kuprash *et al*., 1995)
and TNF-α promoter: 5′-GGGCATGGGAATTTCCCACTCAAGCT-3′ (Kuprash *et al*., 1995).

Oligonucleotides were end labelled with T4 DNA polynucleotide kinase, annealed and separated in native 5% polyacryamide gel. DNA probe (6×10^4 d.p.m.) were used for each binding assay of nuclear extracts as follows: 1 μg of protein was incubated for 15 min at 4°C with the labelled DNA and 1 μg of poly(dI-dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM Tris–HCl pH 7.8, in a final volume of 20 μl. The DNA–protein complexes were separated on native 6% polyacrylamide gels in 0.5 Tris-borate EDTA buffer. For supershift assays, samples were preincubated with the anti-p65 or anti-p50 antibodies (Santa Cruz, CA) 30 min before adding the labelled probe.

Western blot analysis

Cytosolic proteins were subjected to 10% SDS–PAGE, transferred to a nitrocellulose membrane and probed with an anti-IκBα (Santa Cruz, CA), or anti-iNOS antibodies (Sigma). Bound antibodies were decorated with goat anti-rabbit/horseradish peroxidase and blots were developed using the Renaissance blotting detection system (New England Nuclear, Boston, MA).

Indirect immunofluorescence labelling

One hour after treatment with 10 ng/ml of mTNF-α, 3T3 cells were fixed with 2% paraformaldehyde in PBS for 2 min and methanol for 8 min, at room temperature, followed by permeabilizing treatment for 10 min using 0.2% (w/v) Triton X-100 in PBS. Incubation with affinity-purified 1/100 dilution of anti-p65, anti-goat IgG (Santa Cruz) was performed for 1 h at room temperature followed by 30 min incubation with anti-donkey HRP and 30 min with CY3-conjugated anti-HRP (1:400 dilution in PBS, Tween 0.1%, 1% BSA). Coverslips with stained cells were mounted in Mowiol (Hoescht, Germany). Photographs were taken on a Zeiss IM35 microscope.

Contraction experiments

Segments of aortic rings with functional endothelium were mounted 8 h after treating the mice with saline or LPS (40 mg/kg) in a myograph filled with physiological salt solution kept at 37°C and continuously gassed with a mixture of 95% O_2:5% CO_2 (pH 7.4), and mechanical activity was recorded isometrically. Concentration–response curves were constructed by cumulative application of the thromboxane A_2 agonist (9,11-dideoxy-11α, 9-epoxymethano-prostaglandine F) U46619 in aortae.

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Note added in proof

Two very recent publications have described a novel poly (ADP-ribose) polymerase homologue which has been named as PARP-2 (Amé *et al*., 1999; Berghammer *et al*., 1999). Poly (ADP-ribose) polymerase-1 (PARP-1) is the classic 113 kDa PARP homologue.