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Influence of pentoxifylline on fevers induced by bacterial lipopolysaccharide and tumor necrosis factor-α in guinea pigs

Jörg-Michael Goldbach, Joachim Roth, Birgit Störr, Eugen Zeisberger *

Physiologisches Institut am Klinikum der Justus-Liebig-Universität, Aulweg 129, D-35392 Giessen, Germany

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Abstract

In guinea pigs intraperitoneal (i.p.) injections of 50 mg/kg pentoxifylline had no influence on abdominal temperature while higher doses of pentoxifylline caused a hypothermic response lasting for 2–3 h. Administration of 50 mg/kg pentoxifylline 1 h before intramuscular (i.m.) injections of 20 μg/kg bacterial lipopolysaccharide reduced the lipopolysaccharide-induced production of endogenous tumor necrosis factor-α (TNF-α) by 68%. The second phase of lipopolysaccharide-induced fever was significantly attenuated by pretreatment with 50 mg/kg pentoxifylline, a dose which had, per se, no influence on core temperature of guinea pigs. The thermal response of guinea pigs to administration of exogenous TNF-α was not modulated by pretreatment with pentoxifylline. Intra-arterial infusions with 5 μg/kg TNF-α, a dose which yielded the same circulating TNF bioactivity as i.m. injections of 20 μg/kg lipopolysaccharide, induced a biphasic febrile response. The magnitude and duration of TNF-induced fever were the same whether guinea pigs were pretreated with pentoxifylline or with 0.9% saline. The results indicate that endogenous formation of TNF-α may contribute to the development of fever induced by lipopolysaccharide, but is not its only mediator, since the first phase of lipopolysaccharide-induced fever was not altered by the blockade of TNF production.

Keywords: Pentoxifylline; TNF-α (tumor necrosis factor-α); Anti-TNF strategy; Lipopolysaccharide; Fever; TNF bioassay; Radiotelemetry; (Guinea pig)

1. Introduction

The proinflammatory cytokines, interleukin-1 and tumor necrosis factor-α (TNFα) are regarded as principal endogenous mediators of the septic shock syndrome (Dinarello, 1991). For TNF-α such a role was based on the observations that passive immunization against this protein protected experimental animals from the lethal effects of bacterial lipopolysaccharide (Beutler et al., 1985) and that administration of exogenous TNF-α mimicked biological properties of lipopolysaccharide (Tracey et al., 1986). When lipopolysaccharide is administered in sublethal doses, a febrile response rather than a shock-like state is induced in experimental animals (Kluger, 1991) or humans (Burrel, 1994). If TNF-α is the principal mediator of lipopolysaccharide-induced fever, it should fulfil a number of criteria (Kluger, 1991) which can be summarized briefly as follows. In response to injection of lipopolysaccharide, endogenous TNF-α should be released in a temporal pattern consistent with the development of the febrile response; injection or infusion of exogenous TNF-α in amounts comparable to those released under the influence of lipopolysaccharide should evoke a fever; anti-TNF strategies which antagonize production or action of TNF-α should result in an attenuation of fever induced by lipopolysaccharide. Bioactive TNF-α is measurable in the systemic circulation briefly after injection of lipopolysaccharide (Michie et al., 1988a; Cannon et al., 1990; Long et al., 1990; Roth et al., 1993; Jansky et al., 1995). Administration of exogenous TNF-α induces fever although there is controversy (cf., Kluger, 1991, for review), as to whether the febrile response manifests itself under the influence of a pharmacological (Kettelhut and Goldberg, 1988) or of a physiological (Michie et al., 1988b; Roth et al., 1994) dose of this cytokine. Attempts to inhibit TNF activity by means of antisera in experimental animals led to conflicting results (for review: Kluger, 1991), a problem resulting partly from the lack of availability of species-specific TNF antibodies. An experimental alternative to the inhibition of TNF bioactivity is blockade of the endogenous production and release of TNF-α. The phos-

* Corresponding author. Tel.: (49-641) 994-7230; Fax: (49-641) 994-7239; e-mail: Eugen.Zeisberger@physiologie.med.uni-giessen.de

phodiesterase inhibitor, pentoxifylline, has been shown to inhibit the formation of TNF-α in vitro (Strieter et al., 1988; Han et al., 1990) and in vivo (LeMay et al., 1990a; Zabel et al., 1991, 1993), an effect mediated by the increase of intracellular cyclic adenosine-monophosphate (cAMP) levels (Badger et al., 1994). Two studies have been performed to test the suppressive effect of pentoxifylline on TNF production in relation to the febrile response induced by lipopolysaccharide. In one study in human volunteers (Zabel et al., 1991), an intravenous infusion with a total amount of 500 mg pentoxifylline within 4 h resulted in a decrease of lipopolysaccharide-induced circulating TNF-α which was not accompanied by a significant suppression of lipopolysaccharide-induced fever. In rats an intraperitoneal injection of 200 mg/kg pentoxifylline significantly attenuated lipopolysaccharide-induced fever and circulating TNF-α (LeMay et al., 1990a). However, this dose of pentoxifylline per se had a hypothermic effect on body core temperature, probably based on the property of pentoxifylline to induce vasodilation at high doses. In the present study in guinea pigs we therefore tried to characterize the influence of pentoxifylline on lipopolysaccharide-induced fever by means of the following experimental approaches. First, in a dose-response experiment we determined the amount of pentoxifylline which did not alter core temperatures. Second, we tested if this dose of pentoxifylline was effective to reduce endogenous formation of TNF in response to lipopolysaccharide. Third, we investigated if lipopolysaccharide-induced fever was altered by this dose of pentoxifylline. Fourth, we studied if the fever induced by a systemic infusion of exogenous TNF-α, in amounts comparable to those released endogenously after injection of lipopolysaccharide (cf., Roth et al., 1994), was altered by administration of pentoxifylline. The results of these experiments should enable us to state whether modulation of the endogenous formation of TNF-α plays a role in the manifestation of the febrile response induced by bacterial lipopolysaccharide.

2. Materials and methods

2.1. Animals

This study was performed in male guinea pigs with a mean body weight of 370 ± 15 g at the beginning of the experiments. The animals were housed in individual cages at 22°C with a 12/12-h light/dark cycle (light off at 7:00 p.m.).

2.2. Surgery

At least one week before the start of the experimental procedure, some of the animals were chronically implanted with intra-arterial catheters for blood sampling or intra-arterial infusions as described previously (Roth et al., 1993; Roth and Zeisberger, 1995). Briefly, the guinea pigs were anesthetized with 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazin. Polyethylene catheters were inserted through the left carotid artery up to the aortic arch. The distal ends of the catheters were tunnelled subcutaneously to the interscapular region of the back where they emerged through the skin. After implantation, the catheters were flushed with sterile heparinized saline and closed by heating. The catheters were flushed repeatedly twice a week with heparinized saline to prevent hemostasis.

2.3. Experimental protocols

Experiment 1 was performed to determine the dose of pentoxifylline which could be administered without influencing the animals' abdominal temperature. Pentoxifylline (Sigma, St. Louis, MO, USA) was dissolved at concentrations of 20, 40 or 80 mg/ml in sterile pyrogen-free 0.9% saline. Doses of 50, 100 and 200 mg/kg were used for intraperitoneal (i.p.) injections.

Experiment 2 was performed to measure if a dose of 50 mg/kg pentoxifylline significantly reduced lipopolysaccharide-induced production of TNF-α. For this purpose 50 mg/kg pentoxifylline at a concentration of 20 mg/ml or an adequate volume of solvent was injected i.p. 60 min prior to an intramuscular (i.m.) injection of 20 μg/kg bacterial lipopolysaccharide derived from *Escherichia coli* (0111:B4; Sigma). Lipopolysaccharide was dissolved in sterile pyrogen-free 0.9% saline at a concentration of 100 μg/ml and injected into the thigh muscle. In this experiment blood samples (0.6 ml) were collected 60 min before, as well as 60 min and 180 min after, injection of lipopolysaccharide. Blood was slowly (within 1 min) drawn into a sterile syringe via the intra-arterial catheter, put into a polypropylene tube and immediately centrifuged. The blood plasma was stored at −70°C for later determination of TNF.

Experiment 3 was performed to measure lipopolysaccharide-induced fever under the influence of 50 mg/kg pentoxifylline or an adequate volume of solvent. Again, pentoxifylline or solvent was injected i.p. 60 min prior to the i.m. injections of 20 μg/kg lipopolysaccharide.

Experiment 4 was performed to test if TNF-α (Biermann, Bad Nauheim, Germany; specific activity 20 000 U/μg TNF) at a dose of 5 μg/kg dissolved in 2 ml sterile pyrogen-free saline and infused for 30–40 min intra-arterially induced the same circulating levels of TNF-α as i.m. injections of 20 μg/kg lipopolysaccharide. This experiment had already been performed in a previous study (Roth et al., 1994), but was now repeated because we used a new lot of TNF (for details on the procedure of this experiment, cf., Roth et al., 1994; Roth and Zeisberger, 1995).

Experiment 5 was performed to study if fever induced by systemic infusions of exogenous TNF-α was altered

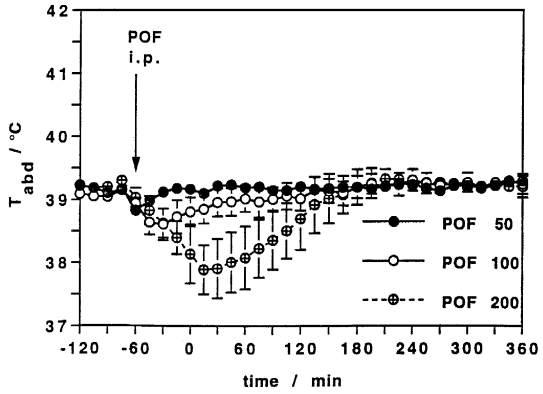

Fig. 1. Changes in core temperature after i.p. injections of guinea pigs with 50, 100 or 200 mg/kg pentoxifylline. Data are presented as means ± S.E.M. ($n = 5$ in each group).

under the influence of pentoxifylline. For this purpose 50 mg/kg pentoxifylline or an adequate volume of solvent was injected i.p. 60 min prior to the start of intra-arterial infusions of 5 μg/kg TNF-α or solvent.

2.4. Measurement of body temperature

Abdominal temperature was measured with battery-operated biotelemetry transmitters (VM-FH-discs, Mini-Mitter, Sunriver, OR, USA) implanted intraperitoneally after placement of the intra-arterial catheter or separately in animals not equipped with catheters. Output (frequency in Hz) was monitored by a mounted antenna placed under each animal's cage (RA 1000 radioreceivers, Mini-Mitter) and multiplexed by means of a BCM 100 consolidation matrix to an IBM personal computer system. A Dataquest IV data acquisition system (Data Sciences, St. Paul, MN, USA) was used for automatic control of data collection and analysis. Body temperature was monitored and recorded at 5-min intervals. Temperature data from 15-min intervals were used for the analysis and graphical documentation.

2.5. Bioassay for TNF

TNF was determined in a bioassay based on the cytotoxic effect of TNF on the mouse fibrosarcoma cell line WEHI 164 subclone 13 (Espevic and Nissen-Meyer, 1986). The WEHI cells were kindly provided by Dr. Stephen Hopkins, University of Manchester, UK. The assay was performed with sterile 96-well microtiter plates. In each well 50 000 WEHI cells were incubated for 24 h with serial dilutions of biological samples or with different concentrations of TNF standard (code 88/532, National Institute for Biological Standards and Control, South Mimms, UK). The number of living cells after incubation was measured with the dimethylthiazol-diphenyl tetrazolium bromide (MTT) colorimetric assay (Gerlier and Thomasset, 1986).

2.6. Evaluation and statistics

In graphs of the thermal responses to injections of pentoxifylline or lipopolysaccharide and to intra-arterial infusions of TNF-α, the mean changes in abdominal temperature were plotted over time. Abdominal temperatures at each time point were expressed as means ± S.E.M. An analysis of variance (ANOVA) for repeated measures followed by Scheffe's post-hoc test was used to compare thermal responses. The calculations were carried out on an Apple Macintosh computer, using the StatView software package (Abacus Concepts, Berkeley, CA, USA). Circulating levels of TNF-α were compared by means of Student's *t*-test.

3. Results

The influence of different doses of pentoxifylline injected i.p. on abdominal temperature of guinea pigs is summarized in Fig. 1.

The injection of 50 mg/kg pentoxifylline had no apparent influence on guinea pigs' core temperatures. After injection of 100 mg/kg pentoxifylline, abdominal temperature tended to fall, and in response to 200 mg/kg pentoxifylline, body temperature was significantly lowered for about 2 h when compared to pre-injection values or to the abdominal temperatures measured after injections of the lower doses of pentoxifylline. For the further experiments the animals were therefore injected with 50 mg/kg pentoxifylline prior to administration of pyrogens or solvent.

Lipopolysaccharide-induced release of TNF-α into the systemic circulation was measured in 2 groups of guinea pigs which received i.p injections of pentoxifylline or an equivalent volume of solvent 60 min before lipopolysaccharide was administered. The results are shown in Fig. 2.

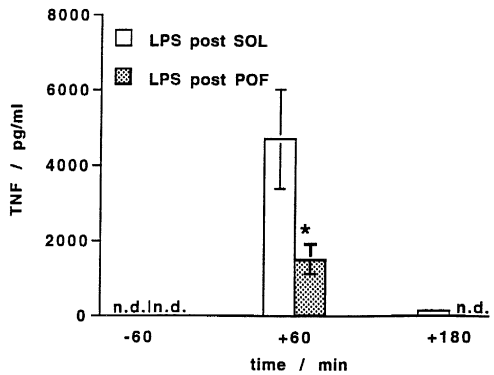

Fig. 2. Bioactive TNF in arterial plasma in response to i.m. injections of 20 μg/kg lipopolysaccharide in guinea pigs pretreated with 50 mg/kg pentoxifylline or solvent ($n = 8$ in each group) 60 min before as well as 60 and 180 min after administration of lipopolysaccharide. Pentoxifylline or solvent was injected i.p., 60 min before lipopolysaccharide was administered. Columns represent means, bars indicate S.E.M.

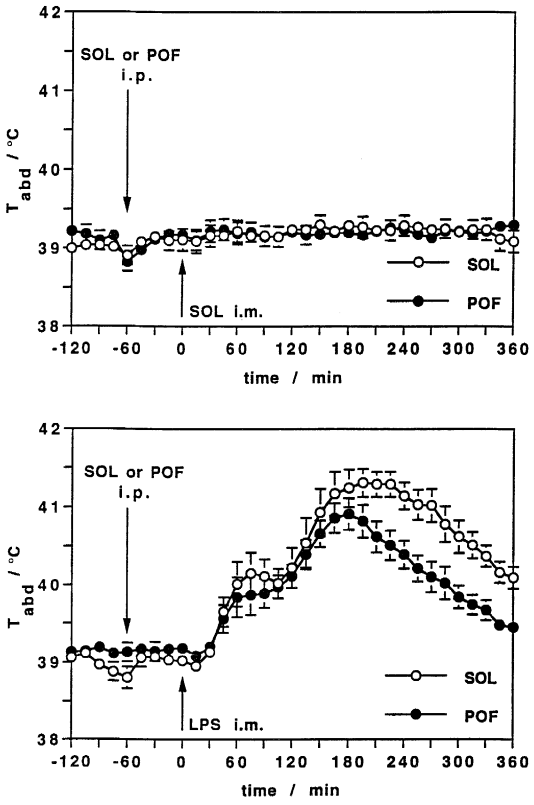

Fig. 3. Changes in core temperature in response to i.m. injections of solvent (upper part) or 20 μg/kg lipopolysaccharide (lower part) in guinea pigs pretreated with 50 mg/kg pentoxifylline or solvent ($n = 7$ in each group). Pentoxifylline or solvent was injected i.p., 60 min before solvent or lipopolysaccharide was administered intramuscularly. Data are presented as means ± S.E.M.

No bioactive TNF-α was detected 60 min before the injection of lipopolysaccharide. At 1 h after intramuscular injection of lipopolysaccharide, when circulating TNF reaches peak activity in guinea pigs (Roth et al., 1993), bioactive TNF corresponding to an amount of 4713 ± 1313 pg/ml was measured in arterial plasma of the control group. This value was significantly reduced to 1517 ± 412 pg/ml in guinea pigs pretreated with pentoxifylline ($P < 0.05$). Three hours after injection of lipopolysaccharide, circulating TNF activity was reduced to 143 ± 57 pg/ml in the control group, while no more TNF was detectable in the plasma of guinea pigs pretreated with pentoxifylline. This result confirmed that, at a dose of 50 mg/kg, pentoxifylline significantly inhibited the endogenous formation of TNF-α in response to lipopolysaccharide. The influence of 50 mg/kg pentoxifylline on lipopolysaccharide-induced fever is shown in Fig. 3.

After administration of pentoxifylline the second phase of lipopolysaccharide-induced fever, between 195 and 360 min after pyrogen application, was significantly attenuated ($F = 10.88$; $P = 0.008$; ANOVA). In the control animals injected with solvent instead of lipopolysaccharide no changes in abdominal temperature were monitored.

In the next experiment we investigated if not only the

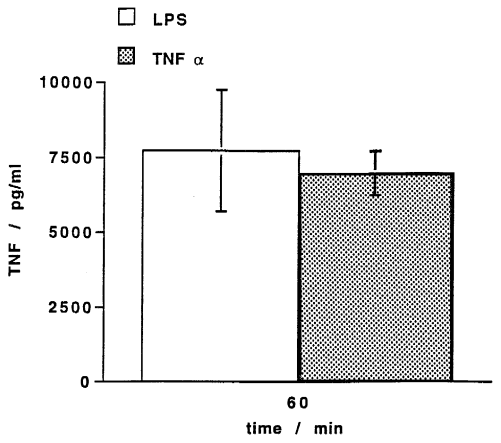

Fig. 4. Bioactive TNF in arterial plasma of guinea pigs, measured 60 min after start of an intra-arterial infusion of 5 μg/kg TNF-α or 60 min after i.m. injection of 20 μg/kg lipopolysaccharide ($n = 6$ in each group). Columns represent means, bars indicate S.E.M.

production of endogenous TNF-α, but also the responsiveness to exogenous TNF-α is modulated by pretreatment with pentoxifylline. For this purpose an amount of 5 μg/kg TNF-α was infused into the carotid artery within 45 min. The circulating level of bioactive TNF-α measured 60 min after the start of infusion was measured and

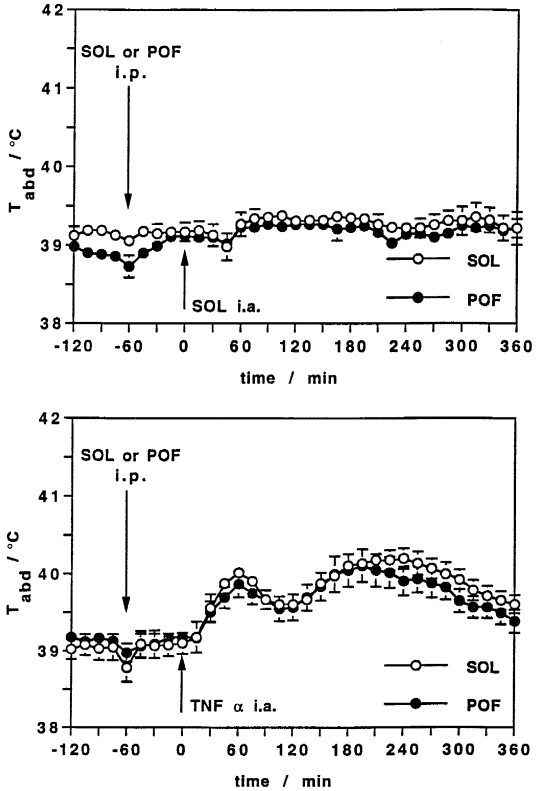

Fig. 5. Changes in core temperature in response to intra-arterial infusions of solvent (upper part) or 5 μg/kg TNF-α (lower part) in guinea pigs pretreated with 50 mg/kg pentoxifylline or solvent ($n = 6$ in each group). Pentoxifylline or solvent was injected i.p., 60 min before the intra-arterial infusion of solvent or TNF-α was started. Data are presented as means ± S.E.M.

compared to the lipopolysaccharide-induced TNF-α peak, also determined 60 min after the administration of lipopolysaccharide. The results are shown in Fig. 4.

The level of circulating bioactive TNF-α was the same 60 min after i.m. injection of 20 μg/kg lipopolysaccharide (7726 ± 2026 pg/ml) as 60 min after the start of an intra-arterial infusion of 5 μg/kg TNF-α (6973 ± 736 pg/ml). Fevers induced by systemic infusions of 5 μg/kg TNF under the influence of a pretreatment with pentoxifylline or solvent are illustrated in Fig. 5.

Intra-arterial infusions of 5 μg/kg TNF-α induced biphasic fevers in guinea pigs, an observation which we had reported earlier (Roth et al., 1994; Roth and Zeisberger, 1995). I.p. injections of pentoxifylline, 60 min before the start of infusion with TNF-α, had no influence on magnitude or duration of the febrile response. Intra-arterial infusions of solvent instead of TNF-α did not induce any changes in abdominal temperature in animals pretreated with pentoxifylline or solvent.

Taken together the results indicate that pentoxifylline inhibits production of endogenous TNF-α and, partly, lipopolysaccharide-induced fever in guinea pigs, while the fever induced by administration of exogenous TNF-α was not modulated by this drug.

4. Discussion

Kasting (1989) listed criteria which should be fulfilled by a substance before it could truely be termed an antipyretic drug. Such a substance should attenuate the febrile rise of body temperature without affecting the body temperature of healthy afebrile subjects. Because of the vasodilator property of pentoxifylline administered in higher doses (LeMay et al., 1990a), it was necessary to determine the dose of pentoxifylline which had, per se, no influence on core temperature. From the results shown in Fig. 1 it becomes obvious that doses of pentoxifylline higher than 50–100 mg/kg indeed induced a hypothermic response in guinea pigs and should therefore not be used for studies dealing with the modulation of fever. In subsequent experiments we confirmed that the dose of pentoxifylline chosen was able to reduce production of endogenous TNF-α by about 68%. The potential of pentoxifylline to reduce or block endogenous TNF formation had protective effects in experimental models of endotoxic shock in some studies (Zabel et al., 1993; Badger et al., 1994) but failed to improve survival in *Candida albicans*-infected mice (Netea et al., 1995). If the dose of bacterial lipopolysaccharide injected into humans or experimental animals does not exceed a certain level, depending on the species-specific sensitivity to lipopolysaccharide, a febrile response with recovery within 8 h is induced rather than an endotoxic shock-like state. We were interested to find if blockade of endogenous production of TNF-α by pentoxifylline would alter the lipopolysaccharide-induced febrile response, since the data available from the literature concerning this point did not completely answer the question (LeMay et al., 1990a; Zabel et al., 1991). As shown in Fig. 3, the second phase of lipopolysaccharide-induced fever was significantly attenuated by pretreatment with 50 mg/kg pentoxifylline. Therefore it seems possible that TNF-α released during the early stage of lipopolysaccharide fever is one of the inducers of release of endogenous mediators that are responsible for the maintenance of the second phase of fever. The first 3 h of lipopolysaccharide-induced fever were not attenuated by blockade of about 68% of the endogenous TNF production. This means that either the remaining 32% of endogenously released TNF or other mediators induced by lipopolysaccharide are able to generate the complete magnitude of the first phase of lipopolysaccharide fever. One candidate for the induction of the early phase of lipopolysaccharide-induced fever is interleukin-1, claimed to be the most important endogenous pyrogen (Dinarello, 1988) and there are studies which have shown the lack of effect of pentoxifylline on interleukin-1 production (Semmler et al., 1993; Netea et al., 1995). The second phase of lipopolysaccharide-induced fever, a time when bioactive TNF-α has already disappeared from the circulation, is significantly attenuated when endogenous TNF formation was blocked by pentoxifylline. TNF-α is known to induce the production of further endogenous pyrogens, for example interleukin-6 (Dinarello, 1991; Roth et al., 1994). After injection of lipopolysaccharide, circulating levels of interleukin-6 are attenuated by treatment with neutralizing antibodies to TNF-α (Fong et al., 1989) or pentoxifylline (LeMay et al., 1990a). Since a contribution of interleukin-6 in the generation and maintenance of lipopolysaccharide-induced fever has been suggested (LeMay et al., 1990b; Blatteis et al., 1990; Rothwell et al., 1991), it seems possible that blockade of TNF-α production, and thereby attenuation of the endogenous release of interleukin-6 and possibly of other cytokines also, is a resasonable explanation for the observed suppression of the second phase of lipopolysaccharide fever. In contrast to our findings, it has been shown that only circulating TNF-α, but not the febrile response, is reduced when pentoxifylline is infused into the circulation of human volunteers injected with lipopolysaccharide (Zabel et al., 1991).

The reason for this discrepancy from our results may be the use of different doses of lipopolysaccharide and pentoxifylline. In the study in humans, lipopolysaccharide was used in nanogram amounts, and pentoxifylline was administered at a dose of about 7 mg/kg. Of course, it is not possible to test higher doses of lipopolysaccharide in humans. The TNF response to the administration of lipopolysaccharide was, however, rather moderate in the human volunteers. Therefore the production of endogenous TNF-α might not have contributed significantly to the development of the febrile response in the human subjects. In studies in rats (LeMay et al., 1990a) and guinea pigs (this paper) administration of higher doses of lipopolysaccha-

ride and pentoxifylline resulted not only in a significant inhibition of TNF formation by pentoxifylline, but also in an attenuation of fever, possibly for the reasons discussed above.

Finally, we were interested to know if the phosphodiesterase inhibitor pentoxifylline interfered not only with the production of endogenous TNF-α, but also with the animals' responsiveness to TNF-α administered exogenously. As shown in Fig. 5 the febrile response to intra-arterial infusions of 5 μg/kg TNF-α was not influenced by pretreatment with pentoxifylline. This result indicates that the development of fever in general is not necessarily influenced by pentoxifylline. This substance seems thus not to interfere at some point in the signal pathway leading to the generation of fever, but only to influence fevers which are, in part, dependent on the formation of endogenous TNF-α.

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