Role of the hepatic function in the development of the pyrogenic tolerance to muramyl dipeptide

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Ferreira, Márcia E. S., Márcio M. Coelho, and Irene R. Pelá. Role of the hepatic function in the development of the pyrogenic tolerance to muramyl dipeptide. Am J Physiol Regulatory Integrative Comp Physiol 281: R162–R169, 2001.—We have demonstrated that the hepatic function may have an important role in the development of tolerance to the pyrogenic effect induced by endotoxin. To further investigate if the role of the hepatic function in the development of tolerance also extends to that induced by other pyrogenic stimuli, we investigated the effect of galactosamine, a specific inhibitor of the hepatic protein synthesis, on the development of tolerance to the pyrogenic effect induced by muramyl dipeptide (MDP) in rats. Pyrogenic tolerance was observed after the second intravenous or intraperitoneal injection of MDP (500 µg/kg), 24 h after the first injection, similar to what was observed with endotoxin. Pyrogenic tolerance was abolished when galactosamine (300 mg/kg ip) was injected simultaneously with MDP (500 µg/kg iv) on the first day. When uridine (600 mg/kg ip) was administered simultaneously with galactosamine (300 mg/kg ip) and the first injection of MDP (500 µg/kg ip), pyrogenic tolerance was again observed after the second injection of the peptidoglycan. In conclusion, the hepatic function may not be important only for the development of tolerance to endotoxin, but also to a totally different pyrogenic stimulus such as MDP.

Although fever represents an important adaptative response (20, 30), the development of tolerance to the pyrogenic effect induced by endotoxin (2, 34, 43), muramyl dipeptide (MDP) (32), tumor necrosis factor-α (TNF-α) (9), or interleukin-1α (IL-1α) (9), or interleukin-1β (IL-1β) (32), or interleukin-6 (IL-6) (28), or interleukin-10 (IL-10) (10) has been observed when these pyrogenic stimuli are repeatedly injected in experimental animals. It has been demonstrated that the tolerance is not restricted to the pyrogenic effect but also extends to the lethality, weight loss, and hypotension induced by some of the previously mentioned inflammatory stimuli (18, 22).

However, the mechanisms involved in the establishment of the pyrogenic tolerance are not fully understood. Both central and peripheral changes have been suggested to explain the development of this phenomenon. Nakamori et al. (27) reported that the production of IL-1β in some circumventricular organs (organum vasculosum laminae terminalis, subfornical area, and postrema area) is markedly reduced in pyrogenic tolerant rabbits. The production of endogenous cryogens may also be involved in the development of pyrogenic tolerance. Cooper et al. (3) observed an increased arginine vasopressin immunoreactivity in the septal area of pyrogenic tolerant guinea pigs, whereas Wilkinson and Kasting (42) demonstrated that the microinjection of a V1 antagonist into the septal area partially inhibited the development of tolerance to the pyrogenic effect of endotoxin. 

In addition to the central changes that may underlie the development of pyrogenic tolerance, peripheral mechanisms may also be important. Beeson (2) and Cooper and Cranston (4) suggested that the tolerance to the pyrogenic effect of endotoxin would result from an increase of its clearance by the reticuloendothelial system resulting in a faster elimination of endotoxin from the circulation. Tolerant animals may also produce a seric component that neutralizes endotoxin, as suggested by Riveau et al. (29) and Warren et al. (41). However, the aspect that has been most investigated to explain the development of tolerance is the production of the pyrogenic cytokines. There are many reports showing that the production of TNF-α, IL-1β, and IL-6 induced by endotoxin is markedly reduced in tolerant animals (6, 25, 33, 37, 45). In addition, Frankenberger et al. (10) demonstrated that IL-10, a cytokine that presents an anti-inflammatory activity, is upregulated in endotoxin-tolerant rats. They also suggested that the pyrogenic tolerance is not a passive reduction of the functions of a tolerant cell but a well-orchestrated response characterized by a reduced production of inflammatory and an increased production of anti-inflammatory cytokines.

The hepatic function has also been proposed as an important element in the development of tolerance to some of the effects induced by endotoxin. Galactosamine, a specific inhibitor of the hepatic protein synthesis (19), abolishes the protection against endotoxic shock induced by previous treatment with TNF-α, IL-1β, and...
interferon-γ (24). Vogels et al. (39) have also suggested that the protective effect conferred by the previous treatment with cytokines against the mortality induced by high doses of endotoxin is mediated by the synthesis of hepatic acute phase proteins (APP). We have recently demonstrated that the hepatic function may also have an important role in the development of tolerance to the pyrogenic effect induced by endotoxin (8). When galactosamine was administered simultaneously with the first injection of endotoxin, the development of tolerance observed after the second injection of the pyrogenic stimulus was abolished.

The exact way the hepatic function contributes to the development of tolerance to the pyrogenic effect induced by endotoxin is not clear, but the synthesis of APP may be involved. Some APP inhibit the production of prostaglandins and fever induced by TNF-α and IL-1β (36). The liver of tolerant animals may also be the source of a seric component that neutralizes endotoxin, as suggested by Warren et al. (41) and Riveau et al. (29). More important, the liver has been found to be the source of a seric component that neutralizes endotoxin from the blood (11, 26, 44). Endotoxin may be processed by hepatocytes and secreted into the biliary zone for rats (28–30).

MATERIALS AND METHODS

Experimental animals. Male Wistar rats (180–200 g) were used. The animals were housed at 24°C and with a 12:12-h light-dark cycle. The animals had access to food and water ad libitum.

Surgical procedures. One week before the experiments, rats were anesthetized with pentobarbital sodium (45 mg/kg ip) and stereotaxically implanted with a stainless steel guide cannula (0.8-mm outer diameter × 15-mm length) according to Paxinos and Watson (28). Each guide cannula was fixed to the skull by an acrylic dental cement attached to two stainless steel screws inserted into the frontal bones of the animals. At the end of the experiments, the position of the cannula was confirmed histologically.

Measurement of body temperature. Colonic temperature was measured with a telemeter (Yellow Spring Instruments) in animals that were conscious and minimally restrained only at the moment of the measurement. Plastic-coated thermocouples were inserted 5 cm beyond the anal sphincter. All animals were trained to accept handling and colonic temperature measurements the day before the experiments. Baseline temperature measurements were obtained 2 h before any treatment. Basal colonic temperature was the average of four measurements. Colonic temperature was determined at 1-h intervals during 6 h after the injection of MDP. All experiments were carried out at the thermoneutral zone for rats (28 ± 1°C) (15) between 8 AM and 5 PM.

Experimental protocols. In the experiments to evaluate the development of tolerance, the pyrogenic stimulus was injected at 24-h intervals. When the effect of galactosamine and uridine on the development of tolerance was evaluated, both drugs were injected simultaneously with MDP in the first day.

Protocol 1 evaluated the time course of the febrile response induced by intracerebroventricular, intravenous, or intraperitoneal injection of MDP. The doses used were 50, 100, 250, and 500 μg/kg for the intravenous or intraperitoneal route, and 50, 250, and 750 ng for the intracerebroventricular route.

Protocol 2 evaluated the febrile response induced by three intracerebroventricular injections of MDP (750 ng) or two intravenous injections of MDP (500 μg/kg) at 24-h intervals.

Protocol 3 evaluated the effect of simultaneous injection of galactosamine (300 mg/kg ip) with MDP (500 μg/kg iv) on the first day on the development of pyrogenic tolerance observed after the second injection of MDP (500 μg/kg iv) 24 h later.

Protocol 4 evaluated the effect of simultaneous injection of galactosamine (300 mg/kg ip) and uridine (600 mg/kg ip) with MDP (500 μg/kg ip) on the first day on the development of pyrogenic tolerance observed after the second injection of MDP (500 μg/kg ip) 24 h later. To reduce the distress of the animals during the injection procedures, as they would receive three injections almost simultaneously, we chose the intraperitoneal route for the MDP injection in this protocol.

Drugs. MDP (N-acetylmuramyl-l-alanyl-l-isoglutamine; ICN Biomedicals), D(+)-galactosamine (2-amino-2-deoxy-D-galactopyranose; Sigma), and uridine (Sigma) were used. All drugs were dissolved in pyrogen-free saline (0.9% NaCl). The concentrations of the solutions were MDP (50, 100, 250, and 500 μg/ml for intravenous or intraperitoneal injections; 25, 125, and 375 μg/ml for intracerebroventricular injections), galactosamine (300 mg/ml), and uridine (600 mg/ml).

Injections. When the intracerebroventricular route was used, a volume of 2 μl was injected over a period of 30 s. When the intraperitoneal or intravenous route was used, a volume of 1 ml/kg was injected.

Statistical analysis. All values are means ± SE. Statistical differences were determined by t-test or one-way ANOVA followed by Duncan's post hoc test using the SPSS statistical software. P < 0.05 was considered statistically significant. Fever index (expressed in °C), i.e., the area under the curve over the 6-h monitoring period, was used for statistical analysis.

RESULTS

Microinjection of MDP (250 or 750 ng) into the lateral ventricle induced a statistically significant increase in colonic temperature (Fig. 1) [1-way ANOVA, F(3,23) = 9.78, P = 0.0004]. Intravenous (Fig. 2) [1-way ANOVA, F(4,35) = 12.52, P < 0.0001] or intraperitoneal (Fig. 3) [1-way ANOVA, F(4,37) = 13.03, P < 0.0001] injection of MDP also induced a dose-dependent increase in colonic temperature. Intravenous or intraperitoneal injection of 100, 250, and 500 μg/kg MDP induced a significant increase in colonic temperature that peaked 2–3 h after injection. The increased colonic temperatures induced by intracerebroventricular injection of MDP lasted longer than that induced by intravenous or intraperitoneal injection.

We next investigated the pyrogenic response induced by repeated intracerebroventricular injections of MDP.
The first injection of MDP induced a significant increase in colonic temperature ($t$-test, $t = 5.52$, $P < 0.0001$). The febrile response induced by the second and third intracerebroventricular injections of MDP, on the second and third day, respectively, was not changed, indicating that the development of pyrogenic tolerance does not occur after three intracerebroventricular injections of this pyrogenic stimulus (Fig. 4). On the other hand, a pyrogenic tolerance was clearly observed after the second intravenous injection of MDP (Fig. 5). The first intravenous injection of MDP (500 $\mu$g/kg) induced a characteristic elevation ($0.8^\circ$C) in colonic temperature ($t$-test, $t = 7.07$, $P < 0.0001$), whereas the second injection, on the second day, did not induce an increase in colonic temperature higher than $0.2^\circ$C (1-way ANOVA, $F(2,20) = 21.45$, $P < 0.0001$).

Next, we investigated if the development of tolerance to the pyrogenic effect induced by MDP, similar to that induced by endotoxin (8), could also be inhibited by galactosamine. Intraperitoneal injection of galactosamine (300 mg/kg) simultaneous with the first injection of MDP (500 $\mu$g/kg iv) did not change the febrile response induced by this pyrogenic stimulus (1-way ANOVA, $F(3,29) = 21.10$, $P < 0.0001$). On the other hand, the development of tolerance observed in the second day, after the second injection of MDP, was abolished in the animals simultaneously treated with galactosamine.

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MDP and galactosamine in the first day [1-way ANOVA, F(3,25) = 13.90, P < 0.0001] (Fig. 6).

Most of the effects induced by galactosamine are reversed by uridine, indicating that they involve UTP depletion (7, 16). Thus we also investigated if the effect of galactosamine on the development of tolerance to the pyrogenic effect induced by MDP could be reversed by simultaneous treatment with uridine. In this protocol,

Fig. 4. A1: time course of increase in Tc induced by intracerebroventricular injection of 750 ng MDP on day 1. ■, Sal (n = 16); and ▲, MDP (n = 7). Basal Tcs were 37.5 ± 0.1 and 37.3 ± 0.1°C for ■ and ▲, respectively. A2: fever index. B1: time course of increase in Tc, induced by intracerebroventricular injection of 750 ng MDP on day 2. ■, Sal – day 1 + Sal – day 2 (n = 10); ▲, Sal – day 1 + MDP – day 2 (n = 6); and ●, MDP – day 1 + MDP – day 2 (n = 7). Basal Tcs were 37.5 ± 0.1, 37.4 ± 0.1, and 37.3 ± 0.1°C for ■, ▲, and ●, respectively. B2: fever index. C1: time course of increase in Tc, induced by intracerebroventricular injection of 750 ng MDP on day 3. ■, Sal – day 1 + Sal – day 2 + Sal – day 3 (n = 4); ▲, Sal – day 1 + Sal – day 2 + MDP – day 3 (n = 6); and ●, MDP – day 1 + MDP – day 2 + MDP – day 3 (n = 7). Basal Tcs were 37.5 ± 0.1, 37.3 ± 0.1, and 37.0 ± 0.1°C for ■, ▲, and ●, respectively. C2: fever index. *Significantly different from Sal-treated group.

Fig. 5. A1: time course of increase in Tc induced by intravenous injection of 500 μg/kg MDP on day 1. ■, Sal (n = 15); and ▲, MDP (n = 6). Basal Tcs were 37.3 ± 0.1 and 37.1 ± 0.1°C for ■ and ▲, respectively. A2: fever index. B1: time course of increase in Tc, induced by intravenous injection of 500 μg/kg MDP on day 2. ■, Sal – day 1 + Sal – day 2 (n = 10); ▲, Sal – day 1 + MDP – day 2 (n = 5); and ●, MDP – day 1 + MDP – day 2 (n = 6). Basal Tcs were 37.4 ± 0.1, 37.2 ± 0.1, and 37.3 ± 0.1°C for ■, ▲, and ●, respectively. B2: fever index. *Significantly different from Sal-treated group. #Significantly different from the group treated with Sal + MDP.
we injected MDP intraperitoneally. Similar to what was observed for the intravenous route, the intraperitoneal injection of MDP induced a febrile response with a similar time course and magnitude, and a pyrogenic tolerance was observed after the second injection. Injection of galactosamine simultaneously with MDP in the first day abolished the pyrogenic tolerance in the second day. However, when uridine was simultaneously injected with galactosamine and MDP in the first day, a pyrogenic tolerance was again observed after the second injection of MDP, indicating that the effect of galactosamine was reversed by uridine (Fig. 7) [day 1: 1-way ANOVA, $F(3,47) = 44.48, P < 0.0001$; day 2: $F(4,47) = 15.65, P < 0.0001$].

DISCUSSION

The results of the present study give further support to the hypothesis that the hepatic function plays an important role in the development of tolerance to the fever induced by different pyrogenic stimuli. Galactosamine, a specific inhibitor of the hepatic protein synthesis, abolished the development of tolerance to the pyrogenic effect induced by MDP, a synthetic peptidoglycan with a structure completely different compared with that of endotoxin.

MDP induced fever after intraperitoneal, intravenous, and intracerebroventricular injections in rats. However, the development of tolerance to its pyrogenic effect was only observed after repeated intravenous or intraperitoneal injections. Three intracerebroventricular injections did not result in the development of pyrogenic tolerance. If further injections would result in the development of tolerance is not clear. Goldbach et al. (14) have also reported that tolerance to the pyrogenic effect induced by TNF-$\alpha$ did not develop after four intracerebroventricular injections of this cytokine in guinea pigs. However, Kozak et al. (21) observed a progressive reduction of the febrile response with repeated intracerebroventricular injections of endotoxin in rabbits that reached significance only after the sixth injection. Thus we cannot exclude the possibility that a tolerance to the pyrogenic effect of MDP would be observed after six or more injections. These results suggest that the rapid development of pyrogenic tolerance depends mainly on peripheral mechanisms, as this phenomenon was clearly observed after the second intravenous or intraperitoneal injection of endotoxin (8) or MDP (present study).

The development of pyrogenic tolerance observed after the second injection of MDP, similar to what was observed with endotoxin, was also abolished when galactosamine was injected simultaneously with MDP in the first day. This result extends the proposal that the hepatic function is important for the development of pyrogenic tolerance to stimuli with different structures. Although it has been demonstrated that the liver plays a major role in the clearance of circulating endotoxin from the blood (11, 26, 44), an observation that could give support to the role of hepatic function in the development of tolerance to this pyrogen, the role of this organ in the clearance of MDP is not known.
Galactosamine specifically inhibits the hepatic function, and this effect has been attributed to its metabolism by the hepatocytes, resulting in the depletion of UTP and an accumulation of UDP-galactosamine (5). As UTP is essential to the synthesis of many macromolecules, the synthesis of RNA, proteins, and glycoprotein by the hepatocytes is reduced by galactosamine. Most of the effects induced by galactosamine are thus reversed by uridine, indicating that they involve UTP depletion. When uridine was administered simultaneously with galactosamine and the first injection of MDP, the pyrogenic tolerance was again observed after the second injection of the peptidoglycan. These results are in agreement with the demonstration that other effects of galactosamine are reversed by uridine and suggest that the effects of galactosamine on the development of pyrogenic tolerance also involve UTP depletion.

The inhibition of hepatic function by galactosamine induces an increased sensitivity of experimental animals to endotoxin (1, 12, 13), TNF-α (1, 23), and IL-1 (40). There is evidence that the protection conferred by some cytokines against endotoxic shock or bacterial suspensions is also mediated by APP produced by the liver (24, 39). Alcorn et al. (1) showed that the acute phase response induced by turpentine, characterized by an elevated production of APP, protects mice against the lethal effect induced by high doses of endotoxin or TNF. In addition, Vogels et al. (39) showed that galactosamine abolishes the production of APP induced by IL-1 in mice and the protective effect induced by this cytokine against the lethality induced by Pseudomonas aeruginosa.

As galactosamine is an inhibitor of hepatic protein synthesis, we could suggest that the synthesis of one or more proteins would be essential for the development of tolerance to the pyrogenic effect induced by MDP and endotoxin. These proteins could inhibit the synthesis or neutralize the action of mediators involved in the genesis of fever, and the treatment with galactosamine, simultaneously with the first injection of MDP or endotoxin, would inhibit their synthesis and consequently the development of pyrogenic tolerance. In support of this hypothesis, it has been proposed that a seric component, probably of hepatic origin, neutralizes endotoxin (29, 41) and that serum with a high concentration of APP reduces the lethality induced by endotoxin (38). Directly related to the phenomenon investigated in the present study, it has been demonstrated that serum amyloid A, an APP produced by the liver, inhibits fever and hypothalamic production of prostaglandin E2 induced by cytokines in mice (36).

Schreiber et al. (35) showed that the maximum plasma concentration of α2-macroglobulin and α1-acid glycoprotein, two APP produced by the liver during inflammation, is reached only 48 h after the injection of turpentine, indicating a delayed production of APP after injection of an inflammatory stimulus. This de-
Hepatic Function in the Pyrogenic Tolerance to MDP

layed production, if it also happens after MDP or endotoxin injection, would help explain why galactosamine did not change the febrile response induced by the first stimulus with MDP (present study) or endotoxin (8) but abolished the pyrogenic tolerance in the second day.

Another possibility to explain the inhibition of development of pyrogenic tolerance in animals treated with galactosamine could be a reduction of MDP clearance from the blood, similar to what has been reported for endotoxin. However, there is no evidence that the liver plays a similar role in the clearance of MDP from the blood, which makes this hypothesis totally speculative. Adding to the discussion on the role of the hepatic function in the development of pyrogenic tolerance, Ivanov et al. (17) have recently suggested that the processes leading to pyrogenic tolerance may be controlled by the vagal innervation of the liver via unknown mechanisms.

In conclusion, the present results support the hypothesis that the hepatic function plays an important role in the development of pyrogenic tolerance. The important aspect raised by the present study is that the hepatic function may not be important only for the development of tolerance to endotoxin, a component of the wall of gram-negative bacteria, but also to a totally different pyrogenic stimulus, both in structure and origin, such as MDP. These results suggest that the hepatic function may contribute to the development of pyrogenic tolerance by different ways and give further support to the important role of the liver in the control of different aspects of the inflammatory response.

Perspectives

It has been demonstrated that there is a lack of cross tolerance between endotoxin and MDP in induction of fever, e.g., animals tolerant to the pyrogenic effect induced by endotoxin develop a febrile response after injection of MDP that is not distinguished from the febrile response of naive animals (31). As the hepatic function seems particularly important for the development of pyrogenic tolerance to both endotoxin and MDP, this allows the suggestion that endotoxin stimulates the synthesis and/or release of hepatic factors that are important for the development of tolerance to its pyrogenic effect, but not to that induced by MDP, and vice versa. If this hypothesis is true, it means that the liver may produce different factors that are important for the development of pyrogenic tolerance and, more important, the factors produced would depend on the nature of the pyrogenic stimulus.

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