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Reflex control of inflammation by the splanchnic anti-inflammatory pathway is sustained and independent of anesthesia

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Martelli DM, Yao ST, Mancera J, McKinley MJ, McAllen RM. Reflex control of inflammation by the splanchnic anti-inflammatory pathway is sustained and independent of anesthesia. Am J Physiol Regul Integr Comp Physiol 307: R1085–R1091, 2014. First published August 27, 2014; doi:10.1152/ajpregu.00259.2014.—Following an immune challenge, there is two-way communication between the nervous and immune systems. It is proposed that a neural reflex—the inflammatory reflex—regulates the plasma levels of the key proinflammatory cytokine TNF-α, and that its efferent pathway is in the splanchnic sympathetic nerves. The evidence for this reflex is based on experiments on anesthetized animals, but anesthesia itself suppresses inflammation, confounding interpretation. Here, we show that previous section of the splanchnic nerves strongly enhances the levels of plasma TNF-α in conscious rats 90 min after they received intravenous LPS (60 µg/kg). The same reflex mechanism, therefore, applies in conscious as in anesthetized animals. In anesthetized rats, we then determined the longer-term effects of splanchnic nerve section on responses to LPS (60 µg/kg iv). We confirmed that prior splanchnic nerve section enhanced the early (90 min) peak in plasma TNF-α and found that it reduced the 90-min peak of the anti-inflammatory cytokine IL-10; both subsequently fell to low levels in all animals. Splanchnic nerve section also enhanced the delayed rise in two key proinflammatory cytokines IL-6 and interferon γ. That enhancement was undiminished after 6 h, when other measured cytokines had subsided. Finally, LPS treatment caused hypotensive shock in rats with cut splanchnic nerves but not in sham-operated animals. These findings demonstrate that reflex activation of the splanchnic anti-inflammatory pathway has a powerful and sustained restraining influence on inflammatory processes.

We recently demonstrated that a powerful anti-inflammatory response is reflexly driven from the central nervous system in rats challenged with systemic endotoxin (intravenous LPS) and that the efferent pathway for this reflex is in the splanchnic sympathetic nerves (20, 21). We term this the splanchnic anti-inflammatory pathway.

Our prior study and those by others investigating the neural reflex control of inflammation have been performed predominantly on anesthetized rodents (4, 21, 24). Despite a general acceptance of the conclusions arising from those studies, the possible confounding role of anesthesia has not been systematically addressed. Anesthesia itself has an anti-inflammatory influence (3, 10, 13, 17, 23, 29, 36), which has been suggested to be mediated by sympathetic nerves (3), although other evidence shows that anesthetics can suppress inflammation by non-neural cellular mechanisms (23, 36). The first aim of the present study was, therefore, to test whether the splanchnic anti-inflammatory pathway is activated in response to endotoxemia independently of anesthesia.

In previous studies, the plasma levels of TNF-α have generally been taken as the main index of the acute inflammatory response to immune challenge (4, 21). TNF-α has been described as a “necessary and sufficient” early mediator of inflammation, because exogenous TNF-α alone can drive inflammation, while anti-TNF-α antibodies can block inflammation (32). So far, reflex activation of the splanchnic anti-inflammatory pathway has been shown to inhibit the acute release of TNF-α up to 90 min after LPS challenge. However, it has not been demonstrated whether the anti-inflammatory actions of the splanchnic sympathetic nerves are sustained over the longer term, nor is it known whether other proinflammatory and anti-inflammatory cytokines are affected. The second aim of the present study was, therefore, to test the actions of the splanchnic anti-inflammatory pathway over a longer timescale (6 h), and to measure its effect on other key cytokines: the proinflammatory IL-1α, IL-1β, IL-6, and IFN-γ, as well as the anti-inflammatory IL-10.

METHODS

Ethical approval. All animal experiments were performed in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health.

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Thirty-eight adult male Sprague-Dawley rats (290–350 g), housed at 22°C on a 12:12-h light-dark cycle, were used for these experiments. At the end of each experiment, animals were killed with an overdose injection of pentobarbital sodium (>100 mg/kg iv; Troy Laboratories, Glendenning, NSW, Australia).

**Conscious animal experiments.** At least 1 wk before the terminal experiment, rats were anesthetized (diazepam 5 mg/kg; ketamine 100 mg ip) and given perioperative analgesia (meloxicam; 1 mg/kg im). The greater splanchnic nerves were exposed by a dorsolateral approach, as described elsewhere (21) and either cut bilaterally just beneath the diaphragm (SplancX group) or left intact (Sham group). Animals were then allowed to recover from surgery.

On the day of the terminal experiment, rats were assigned to four different experimental groups: Sham Vehicle (n = 4; splanchnic nerves intact and injected intravenously with 0.5 ml of sterile saline); SplancX Vehicle (n = 4; splanchnic nerves severed and injected with 0.5 ml iv of saline); Sham LPS [n = 5; splanchnic nerves intact and injected intravenously with 60 μg/kg of LPS from Escherichia coli 0111:B4 (Sigma-Aldrich, St. Louis, MO), dissolved in 0.5 ml of saline]; and SplancX LPS (n = 5; splanchnic nerves cut and injected intravenously with 60 μg/kg of LPS in 0.5 ml of saline). LPS (or vehicle) was injected in conscious rats via the tail vein. The tail of each animal was initially submerged in warm water (~45°C) for 2–3 min to induce peripheral vasodilatation and then injected by means of endovenous angiocatheters (23 G; Becton Dickinson Australia, North Ryde, NSW, Australia). After the injection, animals were put back in their cages, and after 90 min, they were acutely anesthetized by means of isoflurane (5%), and artificial ventilation with oxygen was set at 65–70 inflations/min, adjusting the tidal volume to maintain an end-tidal CO2 concentration between 3.5 and 4.5%; 2–3 ml of blood were transcardially collected. Spleen and a portion of liver were also excised. Blood samples were immediately centrifuged (15 min, 2,000 g), the plasma was separated and stored, together with spleen and liver samples, at −80°C for subsequent analysis. A schema of the experimental timeline is shown in Fig. 1.

**Anesthetized animal experiments.** Anesthesia was induced with pentobarbital sodium (60 mg/kg ip), the animal’s trunk was shaved, and the trachea was cannulated. Anesthesia was then maintained for the duration of surgery by 2% isoflurane in pure oxygen, delivered by artificial ventilation (rodent ventilator, Ugo Basile, Italy), before being replaced by urethane for the experiment proper (see below). Artificial ventilation was set at 65–70 inflations/min, adjusting the tidal volume to maintain an end-tidal CO2 concentration between 3.5 and 4.5% throughout the experiment. The right femoral artery and vein were cannulated for monitoring blood pressure and intravenous administration of drugs, respectively. A water-perfused Silastic jacket was positioned around the animal to maintain its body temperature around 37°C. Core temperature was measured by a thermocouple inserted 5 cm into the rectum. When preparatory surgery was complete, anesthesia with isoflurane was gradually withdrawn and replaced by urethane at 1.0–1.2 g/kg iv, and artificial ventilation with oxygen was maintained for the rest of the experiment. Supplementary doses of urethane (10% of original dose) were given as needed to maintain a stable plane of anesthesia.

Animals were subdivided into two experimental groups, in which the greater splanchnic nerves were exposed and either cut bilaterally (SplancX group) or left intact (Sham group). LPS (60 μg/kg; n = 6 animals/group × 2 groups), or saline (n = 4 animals/group × 2 groups) was injected intravenously at time zero. Arterial blood samples (0.8 ml) were collected at baseline (−10 min) and at +90, +180, +270, and +360 min. After each sample, the volume removed was replaced with intravenous saline. Blood samples were immediately centrifuged (15 min, 2,000 g), and the plasma was stored at −80°C (see Fig. 1 for a schema of the experimental timeline).

**Cytokine and corticosterone measurements.** In the conscious animal experiments, plasma samples from all animals were assayed for TNF-α by ELISA (R&D Systems, Minneapolis, MN). Spleen and liver were also assayed for TNF-α but only in LPS-challenged animals. For spleen and liver preparation, frozen tissue samples were weighed and placed in homogenization buffer (PBS, containing a protease-inhibitor cocktail, Sigma-Aldrich; 0.5% Triton X-100; pH 7.2; 4°C) at a ratio of 100 mg of tissue per milliliter. Samples were homogenized and subjected to one freeze-thaw cycle and then sonicated for 5 min. The final homogenate was centrifuged at 12,000 g for 10 min. Tissue supernatants were separated and used for TNF-α determination.

Plasma samples from LPS-injected animals in the second set of experiments (anesthetized animals) were also assayed for IL-6, IFN-γ, IL-1α, IL-1β, and IL-10 using Bio-Plex kits with magnetic beads (Bio-Rad, Gladesville, NSW, Australia). The same samples were also assayed for corticosterone by ELISA (Abnova, Jhongli, Taiwan). Blood samples from the saline-injected animals in the second experimental series (anesthetized animals) were not assayed.

**Statistical analysis.** For the conscious animal experiments, plasma TNF-α levels were compared between experimental groups (Sham LPS and SplancX LPS) nonparametrically by the Mann-Whitney U-test. Plasma and liver TNF-α were compared between Sham LPS and SplancX LPS by t-test.

Data from anesthetized animal experiments were analyzed using a repeated-measures two-way ANOVA. For mean arterial pressure (MAP), core body temperature (T Core), and heart rate (HR), data from the two treatment categories (LPS and saline) were analyzed separately. In each case, the two factors considered were time and experimental group (Sham or SplancX). Post hoc comparisons were performed by the Bonferroni test.

Cytokine and corticosterone measurements were only performed on LPS-injected animals. The two factors considered for the two-way repeated-measures ANOVA were again time and experimental group.
Post hoc comparisons were made by the Bonferroni test. $P$ values <0.05 were considered significant in all experiments.

**RESULTS**

**Conscious animal experiments**—effect of splanchnic nerve section on TNF-α levels in LPS-challenged rats. Sham and SplancX animals recovered in a similar way from the surgery, showing an average weight increase of 34 g and 33 g, respectively, after 1 wk. Plasma levels of TNF-α at 90 min after intravenous injection of saline were undetectable in control animals from both the Sham and SplancX experimental groups (Fig. 2A). By contrast, there was a strong increase in plasma TNF-α in both experimental groups injected with intravenous LPS. Compared with sham-operated animals, the levels of plasma TNF-α at 90 min after LPS injection were substantially higher in the animals whose splanchnic nerves had been cut ($P = 0.008$; Fig. 2A). TNF-α levels in the spleen (Fig. 2B) and liver (Fig. 2C) followed the same trend observed for plasma samples, being higher in SplancX group than in the Sham group ($P = 0.007$ and $P = 0.006$, respectively).

**Anesthetized animal experiments:** effect of splanchnic nerve section on body temperature, heart rate, and blood pressure in LPS-challenged rats. Over the 6 h of the experiment, there was no significant change in T Core, HR, or MAP in the saline-treated control animals of either experimental group (SplancX and Sham) (Fig. 3).

LPS treatment caused a rise in body temperature in both Sham and SplancX experimental groups. The increase in T Core depended on time ($P < 0.001$) but not on the experimental group (Fig. 3).

LPS treatment also increased HR in both Sham and SplancX experimental groups. The increase in HR also depended on time ($P < 0.001$) but not on experimental group (Fig. 3).

LPS induced changes in MAP (Fig. 3), and in this case, those changes depended on both time ($P < 0.001$) and experimental group ($P = 0.002$). The time $\times$ group interaction was also significant ($P < 0.001$). As can be seen in Fig. 3, there was an early (2–3 h) increase in MAP in the Sham group, but this then returned to baseline. In the SplancX group, however, MAP fell to levels significantly below baseline from 4 h onward and did not recover (Fig. 3).

**Anesthetized animal experiments:** effect of splanchnic nerve section on plasma cytokine levels in LPS-challenged rats. From undetectable levels at baseline, intravenous administration of LPS induced a release into the bloodstream of all of the investigated cytokines (Fig. 4). Plasma TNF-α, as expected from previous studies (35), peaked at 90 min, but then returned to undetectable levels after 180 min. At 90 min, the levels of TNF-α were higher in SplancX group than in the Sham group ($P < 0.001$), confirming our previous findings (21) (Fig. 4).

IL-6 was also released in response to endotoxemia, but rose more slowly than TNF-α. In the Sham group, it peaked at 180 min after the injection of LPS, while in the SplancX group, it increased steadily throughout the experiment. Plasma IL-6 levels were significantly higher in SplancX at 270 ($P = 0.05$) and 360 min ($P < 0.001$), with respect to Sham animals (Fig. 4).
state confidently that the inflammatory reflex triggered by endotoxemia is not an artifact due to anesthesia, but functions perfectly well in conscious animals.

This finding on conscious rats and those of our previous study on anesthetized rats (21) demonstrated that the splanchnic anti-inflammatory pathway mediates a reflex response to acute endotoxemia, but only showed this over the short term (90 min) and only for one key mediator, TNF-α. Because of those limitations, we now have measured the effects of splanchnic nerve section in animals for 6 h after the intravenous injection of LPS.

Confirming previous studies (8), we observed that cutting the greater splanchnic nerves does not affect the rise in body temperature after intravenous LPS. Nor was the HR response to LPS affected by splanchnic nerve section (Fig. 3). Interestingly, we found that LPS induced a distinct hypotensive effect at 4, 5, and 6 h after injection only in rats belonging to the SplancX group. The surgical procedure alone was not responsible for the dramatic drop in blood pressure, since it did not occur in saline-treated animals. Three possible mechanisms may contribute to the hypotension. First, the inflammatory reaction of the SplancX animals was stronger, as assessed by the levels of several inflammatory cytokines (discussed below). This could have been sufficient to precipitate hypotensive shock in SplancX rats (33), while the level of inflammation in the Sham rats remained below the threshold for hypotensive shock. The second possible reason is hemodynamic. Cutting the splanchnic nerves removes vasomotor tone from a large vascular bed, whose baroreceptor-mediated compensatory vasocostriction is thereby disabled. Removing sympathetic tone to the spleen may also lead to a fall in blood volume (11), amplifying the hypotensive effect of LPS. Third, sectioning the splanchnic nerves could have diminished the LPS-induced rise in circulating noradrenaline (37), and this may have contributed to the hypotensive effect.

Most importantly, these longer-term experiments allowed us to follow the more slowly responding cytokines and confirmed that the effect of the splanchnic anti-inflammatory pathway is not simply transient. As have others (35), we found that the TNF-α response to LPS resolved within ~3 h. But splanchnic nerve section then enhanced plasma levels of IL-6 and IFN-γ, two other key proinflammatory mediators, 4.5 and 6 h after the injection of LPS. This confirms that “endogenous”, reflexly generated activity in the splanchnic nerve inhibits the release of those proinflammatory cytokines. This result extends previous findings made from “exogenous” activation of sympathetic nerves. Straub et al. (28) found that electrical stimulation of sympathetic nerve terminals in superfused spleen slices taken from arthritic mice inhibited the release of IL-6 and IFN-γ. In contrast, Kees et al. (16) found that electrical stimulation of the sympathetic nerves to the perfused rat spleen caused no reduction in IL-6 response to LPS, although they did find that the TNF-α response was suppressed. In that study, data were collected for 4 h after LPS challenge. Comparison with our present finding suggests that 4 h may have been too early to see the effect of sympathetic nerves on IL-6 release.

Interestingly, the effects of the splanchnic anti-inflammatory pathway on the anti-inflammatory cytokine IL-10 were opposite to those observed on TNF-α. Cutting the greater splanchnic nerves reduced the release of IL-10 at its peak 90 min after the immune challenge. This provides a direct demonstration of

**DISCUSSION**

Our first new finding is that prior section of the splanchnic sympathetic nerves produces a powerful enhancement in plasma TNF-α levels of endotoxemic rats in the absence of anesthesia (Fig. 2A). This confirms and extends our previous discovery that a potent neural reflex inhibits excessive release of this key early mediator of inflammation. Critically, it removes an important potential confound—the anti-inflammatory action of anesthesia (3), discussed below. We can now

**Fig. 3.** Physiological responses to LPS in anesthetized rats. Changes of core body temperature (T core; °C), heart rate (HR; bpm) and mean arterial pressure (MAP; mmHg) in rats subjected to bilateral splanchnic nerve section (SplancX, solid line) or sham-operated (sham, dashed line). Animals were injected intravenously with saline (left) or 60 μg/kg LPS (right) and followed for 6 h. Results are expressed as means ± SE. Data were analyzed using a repeated-measures two-way ANOVA followed by Bonferroni post hoc test. *P < 0.05 compared with baseline levels. #: # # # # # # # # # # * P < 0.05 compared with sham group.

4) IFN-γ behaved similarly to IL-6: SplancX animals had significantly higher levels of IFN-γ at 270 (P = 0.043) and 360 min (P = 0.008) (Fig. 4).

Plasma IL-1α levels peaked at 90 min but revealed no significant difference between Sham and SplancX experimental groups at any time point. Plasma IL-1β also peaked at 90 min and remained above baseline for the rest of the experiment in both Sham and SplancX groups. At 90 min (but not later), the IL-1β levels in the SplancX group were lower than in the Sham group (P < 0.001; Fig. 4).

Plasma levels of the anti-inflammatory cytokine IL-10 peaked in both experimental groups at 90 min and remained above baseline for the rest of the experiment. At 90 min (but not later), IL-10 plasma levels were significantly lower in the SplancX experimental group than in the Sham group (P < 0.001; Fig. 4).

Plasma corticosterone levels were not different between the SplancX and Sham experimental groups at any time point (Fig. 4).
inferences made from indirect evidence on the basis of stress (6, 7): activation of sympathetic nerves enhances the LPS-induced IL-10 production. We now show that this applies to reflex activation of the splanchnic sympathetic nerves. Recently, Harden et al. (12) have shown that pretreatment of rats with antibodies to IL-10 increased their TNF-α response (at 90 min) and their IL-6 response (at 6 h) to LPS. Therefore, it is likely that both the reduced IL-10 and the increased TNF-α levels at 90 min in SplancX rats were related and that both contributed to the enhanced inflammatory response measured at 6 h after LPS treatment.

Surprisingly, the splanchnic anti-inflammatory pathway does not seem to influence the levels of IL-1α released in response to endotoxemia (Fig. 4), and the effect on the proinflammatory cytokine IL-1β was opposite to our expectation, in that cutting the splanchnic nerves induced a decrease in the peak levels of IL-1β 90 min after LPS injection (though not thereafter). The implications of this are unclear. Nevertheless, the final effect at 6 h was a net increase in only proinflammatory cytokines (IL-6 and IFN-γ).

Since the spleen is a major organ mediating inflammatory responses to LPS (14), and splenic nerves have been implicated in the neural control of that inflammation (25, 34), it is conceivable that the primary target organ of the splanchnic anti-inflammatory pathway is the spleen. Consistent with this, we recently found [as have others, e.g., (18)] that LPS causes a sustained increase in the efferent activity of the splenic nerve, and we went on to show that this activity is driven by the splanchnic nerves (5, 21). The anti-inflammatory neural activity is presumed to be directed to macrophages and natural killer cells located in the white pulp of the spleen and to be mediated by α2 adrenergic receptors that bind the noradrenaline released from the sympathetic noradrenergic terminals (9, 15, 16, 34). While our data support the involvement of the spleen (splenic TNF-α levels in LPS-treated rats were enhanced after splanchnic nerve section; Fig. 2B), they do not exclude the involvement of other organs supplied by the greater splanchnic sympathetic nerves. Indeed, TNF-α levels in the liver were also exaggerated if the splanchnic nerves were previously cut.
(Fig. 2C). The involvement of other tissues innervated by the splanchnic nerves remains to be tested.

It should be noted that this splanchnic anti-inflammatory pathway is quite distinct from the "cholinergic anti-inflammatory pathway" (31), in that, it is reflexly activated by LPS and does not require the vagus nerves (21).

Conclusions. The splanchnic anti-inflammatory pathway is the efferent motor pathway of the inflammatory reflex. It is activated reflexly in both conscious and anesthetized endotoxin- and, its dampening effect on the general inflammatory state of the organism is sustained for at least 6 h.

Limitations. Our investigation was restricted to the early stages of inflammation (the first 6 h). Although the profiles of IL-6 and IFN-γ release in SplanC and Sham animals appear to be diverging at the end of the sampling period, further experiments will be needed to determine the chronic effects of splanchnic nerve section.

Second, we only studied the function of the splanchnic anti-inflammatory pathway in endotoxemia induced by intravenous injection of LPS. Further investigations need to be undertaken to understand the role of the inflammatory reflex in other immune challenges, such as those induced by gram-positive bacteria or viruses. These may differ, as shown by the divergent consequences of ablatting abdominal sympathetic nerves on the dissemination of gram-negative bacteria (decrease) or gram-positive bacterial (increase) in mice (27).

Perspectives and Significance

The body's responses to injury and infection have been honed by a long evolutionary history. It is biologically important for the powerful processes of inflammation to be kept under control, because if unchecked, they can be lethal. On the other hand, they must be allowed to proceed sufficiently to respond to the challenge. The CNS plays a major role in regulating the strength of inflammatory responses, and it engages the splanchnic anti-inflammatory pathway to do so early during the process, before slower mechanisms, such as glucocorticoids have a chance to act. The present findings show that this neural mechanism is not simply a transient effect that is dissipated or subsumed by other mechanisms after the first couple of hours. The splanchnic anti-inflammatory pathway's action is sustained for at least several hours. Whether it persists over days or weeks remains to be determined. If so, it may prove highly relevant to the body's endogenous response in conditions such as sepsis and neoplasia. On the other hand, an inadequate response of this pathway might contribute to inflammatory diseases. Localizing the efferent pathway to a nerve, which can be cut or perhaps stimulated, opens up these possibilities to direct study.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


