HEME OXYGENASE-1 INDUCTION BY COBALT PROTOPORPHYRIN ENHANCES FEVER AND INHIBITS PYROGENIC TOLERANCE TO LPS

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Abstract

Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the degradation of heme and regulates its availability for new synthetized hemeproteins, especially cyclooxygenases, NO synthases and cytochrome P450. Moreover, HO-1 activity modulates synthesis of cytokines and prostaglandins. All of these factors are well-defined components of fever and pyrogenic tolerance mechanisms. We examine the effect of HO-1 induction and activation with cobalt protoporphyrin (CoPP) on changes in body temperature (Tb) and plasma levels of interleukin-6 (IL-6), prostaglandin E_2 (PGE_2) and HO-1 protein in the course of these processes. Intraperitoneally (i.p.) pretreatment of rats with CoPP (5 mg kg^{-1}) significantly accelerated and enhanced the early stage of lipopolysaccharide (LPS) induced fever, but also shortened post fever recovery to normal temperature. Pretreatment with CoPP significantly potentiated the increase in plasma IL-6, PGE_2 and HO-1 levels 4 h after LPS administration. Furthermore, induction of HO-1 prevented the development of pyrogenic tolerance to repeated injections of LPS. Based on these data we conclude that heme oxygenase-1 may act as a physiological regulator of the febrile response intensity to bacterial infections. This may provide new insights into the physiological roles of this enzyme and convince to monitoring HO-1 levels as a biomarker for therapeutic interventions.

Key words

fever, pyrogenic tolerance, lipopolysaccharide, interleukin-6, prostaglandin E_2, heme oxygenase

1. Introduction

Fever defined as state of elevated core temperature is often, but not necessarily, part of the defensive responses of organisms to the invasion by live (microorganisms) or inanimate matter recognized as pathogenic or alien by the host. Substances that after entering the inner environment of a multicellular organism cause fever are called exogenous pyrogens (Glossary of terms for thermal physiology, 2001). Among them lipopolisaccharide (LPS, endotoxin) is the agent most frequently used in studies (Blatteis et al., 2000). Released LPS provokes wide spectrum of biological activities and is considered to be the most powerful microbiological stimulant of immune and non-immune (endothelial) cells (Gill et al., 1998). In response to injection of LPS animals develop fever triggered by the release of cytokines (endogenous pyrogens). Cytokines, especially interleukin-1β (IL-1β) and IL-6 are involved in stimulation of acute phase protein synthesis, activation of hypothalamic-pituitary-adrenal (HPA) axis, as well as induction of other cytokines (Kluger, 1991). The pyrogenic actions of these signaling proteins require however the activation of arachidonic acid cascade and release of the cyclooxygenase-dependent metabolites of arachidonate including prostaglandin E_2 (PGE_2) (Kozak et al., 2006). PGE_2 is considered to be a key mediator of fever, acting downstream to the endogenous pyrogens, and is ultimately responsible for the upward resetting of the set point of thermoregulation (Blatteis et al., 2005). Repeated injections of LPS result in progressive attenuation of its pyrogenic effects thus reflecting the development of pyrogenic tolerance (Roth et al., 1994). The mechanism of pyrogenic tolerance is complex and still has not been completely solved but is mainly manifest as reduced ability of macrophages to release endogenous mediators, especially cytokines (Zeisberger and Roth, 1998). The lack of cross tolerance between different pyrogens and the fact that tolerance state can be broken using higher dose of the same pyrogen indicates, that the process should be interpreted as adapted immune response, rather than a state of simple hyporesponsiveness to LPS (Soszynski, 2002).

Heme oxygenase is a stress-responsive enzyme, that degrades heme into equimolar amounts of carbon monoxide (CO), free iron and biliverdin, which is rapidly converted to bilirubin (Otterbein et al., 2000). The main biological function of HO is to avoid the accumulation of highly deleterious free heme, but several aspects of HO biology are likely contribute to its broad effects against large spectrum of diseases and restoring homeostasis (Soares and Bach, 2009). To date, three isoforms of heme oxygenase have been identified (inducible form HO-1 and two constitutively expressed: HO-2, and HO-3) (Wagener et al.,

1999). HO-1 is one of the most important among the acute phase proteins and is induced under the influence of inflammatory and pyrogenic stimuli, including LPS and cytokines (Ryter et al., 2006). Up-regulation of HO-1 leads to reduction in cellular heme and elevation of CO which affects several proteins. It's because heme provides the catalytic domain for nitric oxide synthases (NOS), soluble guanylate cyclase (sGC), monooxygenase enzymes (i.e., cytochrome p-450 family) and cyclooxygenases (Tsiftsoglou et al., 2006). All above factors are crucial for developing both fever and pyrogenic tolerance. Furthermore, CO can bind to the heme moiety of these hemeproteins and cause either enzyme activation or inhibition (Maines, 1997). It was also found, that this diatomic molecule, released during heme degradation has a pyretic effect. Over the years, more evidence has been growing in support of the importance of CO as neuromodulator in central nervous system (CNS) involved in the control of body temperature and fever (Steiner and Branco, 2000). Raffaini and colleagues suggested that also the development of pyrogenic tolerance to LPS is associated with decreased production of CO in the CNS (Raffaini et al., 2006).

The aim of present study was to investigate the effect of HO-1 induction and activation by CoPP on body temperature and plasma IL-6, PGE_2 and HO-1 concentration during fever and pyrogenic tolerance to LPS.

2. Materials and methods

2.1. *Animals and body temperature measurements*

Male rats [Strain: Wistar Crl: WI(Han)] aged 8-12 weeks and weighing from 250 g to 300 g were purchased from the Experimental Medicine Centre Medical University of Bialystok (Poland) and allowed to acclimatize for 10 days before starting the experiments. Animals were kept individually in a room at constant relative humidity (60±5%), temperature (24±1 °C), with a 12:12 h light - dark photoperiod, with lights on at 7:00 h. Rodent laboratory food and drinking water were provided *ad libitum*. All procedures were approved by the Local Bioethical Committee for Animal Care (permission No. 10/2011).To monitor core body temperature (Tb) all animals were implanted intra-abdominally with temperature-sensitive miniature biotelemeters PhysioTel® model TA10TA-F40 (Data Sciences International, St. Paul, MN, U.S.A) under sterile condition (for details see Wrotek et al., 2011). All surgical procedures were done at least ten days before the start of experiments.

2.2. Reagents and Injections

LPS derived from *Escherichia coli* (Sigma-Aldrich St. Louis, MO, U.S.A) was dissolved in sterile 0.9% sodium chloride (saline) and injected intrapertineally (i.p.) at a dose of 50 µg kg^{-1} to provoke fever. Pyrogenic tolerance to LPS was induced by three consecutive daily injections of LPS at the same dose.

Cobalt protoporphyrin (MP Biomedicals, Santa Ana, CA, USA) was dissolved in sodium hydroxide, neutralized with hydrochloric acid and diluted with Phosphate-Buffered Saline (PBS) to a final volume and pH of 7.4. Because of light sensitivity, CoPP was light protected, and freshly made before injection. For the induction of HO-1, animals were pretreated with CoPP (5 mg kg^{-1} i.p.) 24 h before the administration of LPS. The composition of the CoPP-dissolving mixture had no significant effects on Tb as well as on the plasma IL-6, PGE_2 and HO-1 concentrations determined by ELISAs. Therefore saline was used as a control in experiments.

Indomethacin (Sigma-Aldrich) was prepared as an aqueous sodium solution in 0.01 M anhydrous sodium carbonate as described elsewhere (Kozak et al., 1994). Indomethacin was injected intraperitoneally (i.p.) at dose of 10 mg kg^{-1}. This dose inhibited a fever in rats treated i.p. with lipopolysaccharide at a dose of 50 µg kg^{-1}.

HO-1 antibody (rabbit IgG anti-HO-1; Enzo Life Sciences, Farmingdale, NY, U.S.A) was diluted with PBS and injected i.p. in a dose of 25 µl/rat 1 h prior to CoPP. Rabbit IgG was used as control injection and caused no significant changes in Tb (data not presented).

2.3. Blood collection and ELISAs

Blood was collected from anesthetized rats by cardiac puncture into the solution of ethylenediamine tetraacetic acid (EDTA, MP Biomedicals). Plasma was immediately separated by a centrifugation 20 min 1000 x g within 30 min of collection. All samples were kept frozen at -20ºC until assay. IL-6 and PGE_2 levels were determined by an ELISA kits from R&D Systems (Minneapolis, MN, U.S.A). Indomethacin (in a ratio of 0.1 mL of 0.01 M indomethacin per 1 mL of blood) was added to the blood samples used for PGE_2 measure. HO-1 levels were determined using EIA kit from Enzo Life Sciences. Wells were read with an ELISA reader (model Synergy HT; BioTek Winooski, VT, U.S.A).

2.4. Data analysis

Values are reported as means ± standard error mean (S.E.M.). Five-minute temperature recordings were pooled into 20 min averages for presentation. ANOVA with repeated

measures followed by t-test was used to determine differences among groups with the level of significance set at $p<0.05$.

3. Results

3.1. Effect of anti-PGE_2 immunoglobulin and indomethacin on CoPP-induced increase of Tb in rats

Body temperature of saline-injected rats, after a brief 30-min elevation at the time of injection and handling, returned to a pre-injection level (*Fig. 1*). 12-h average daytime Tb of rats treated i.p. with the sterile 0,9% NaCl (37.23±0.03 °C) did not differ from non-treated rats (37.23±0.04 °C, data not shown). *Fig. 1* demonstrates that injection of CoPP (5 mg kg^{-1} i.p.) triggered a significant rise in Tb lasting at least 8h, with average 38.29±0.10 °C measured between fifth and eleventh hour post-injection and preceded by initial drop of about 0.5 °C lasting 3 h. 24 h post-injection a Tb of the animals returned to normal. To determinate whether this elevation of Tb was HO-1 and/or COX-dependent we have used COX inhibitor indomethacin and anti-HO-1 immunoglobulin. Indomethacin injected at a dose 10 mg kg^{-1} immediately before CoPP inhibited both, the drop and rise in Tb observed in CoPP-only treated animals. Average temperature between fifth and eleventh hour post-injection (37.58±0.05 °C) was significantly lower than in animals treated with CoPP ($p<0.05$). A stock solution of rabbit-anti-rat HO-1 immunoglobulin was injected in a dose of 25 µg/rat i.p. an hour before CoPP. HO-1 antibodies significantly inhibited elevation of Tb provoked by the injection of CoPP ($p<0.05$). 6-h average Tb between fifth and eleventh hour post-injection of rats treated with antibodies and CoPP was 37.55±0.06 °C.

Figure 1

3.2. Effect of CoPP on endotoxin fever in rats

Intraperitoneal injection of the saline suspension of *E. coli* LPS at a dose of 50 µg kg^{-1} induced biphasic fever in the rat as can be seen on *Fig. 2*. Fever started within 2 hours post-injection, and the first peak of the Tb rise (38.20±0.09 °C) was reached and maintained during the next 1.5 h. The second peak of fever (38.59±0.11 °C) was reached within 4 h post-injection. Then, a 6 h lasting gradual decrease of Tb towards normal was observed. Pre-treatment of rats with CoPP (5 mg kg^{-1} i.p.) significantly augmented and accelerated fever response to LPS injected after 24 h. The first peak of the Tb rise was reached at 2 h post-injection (38.81±0.13 °C), the second peak was however delayed (38.24±0.09 °C after 320

min) in compare to animals treated with saline a day before LPS. Furthermore, febrile response to LPS lasted 2 h shorter in CoPP-pretreated animals indicating that HO-1 can also participate in post fever recovery to normal temperature.

Figure 2

3.3. Effect of CoPP on pyrogenic tolerance to LPS in rats

A significant pyrogenic tolerance was observed after the second and third injection of LPS (*Fig. 3A*). In contrast to day 1, the injection of LPS on days 2 and 3 evoked only a monophasic rise in Tb. Rats secondly and thirdly injected with LPS responded also with much smaller increases of Tb (38.07±0.1 °C after 2nd and 37.81±0.18 °C after 3rd injection in comparison to 38.59±0.11 °C after the first injection ($p<0.05$)). Animals pretreated with CoPP also showed weakened (with maximum Tb reaching 38 °C) but extended febrile response, that didn't differ after second and third LPS injection (*Fig. 3B*). Furthermore, maximum value of the Tb rise, reached after 2 h, was maintained by the next 3.5 h revealing lack of pyrogenic tolerance to LPS.

Figure 3

3.4. Effect of CoPP on plasma IL-6 and PGE_2 levels during fever and pyrogenic tolerance in rats

The first injection of LPS (50 μg kg^{-1} i.p.) triggered a significant increase in plasma IL-6 (1836±253 pg mL^{-1}) and PGE_2 (1527±94 pg mL^{-1}) levels after 4 h compared to control animals injected with saline (respectively 56±32 pg mL^{-1} IL-6 ($p<0.05$) and 624±95 pg mL^{-1} PGE_2 ($p<0.05$)) as can be seen on *Fig. 4A and B.* Animals pre-treated with CoPP responded to first dose of LPS with significant increase in PGE_2 (1894±111 pg mL^{-1}) and twice as high increase in IL-6 (4325±270 pg mL^{-1}) compared to rats injected with LPS 24 h after saline ($p<0.05$). Three daily consecutive injections of LPS at the same dose led to almost complete reduction in IL-6 plasma concentration (174±27 pg mL^{-1}) and also to the reduction by half in PGE_2 concentration (869±196 pg mL^{-1}) in animals non-treated with CoPP. We have seen that in animals pre-treated with CoPP, PGE_2 level after third LPS injection was even higher than after the first dosage (2221±138 pg mL^{-1}) ($p<0.05$). Administration of CoPP before three daily injections of LPS did not, however, affect the decrease in plasma IL-6 concentration (109±15 pg mL^{-1}).

Figure 4

3.5. Effect of CoPP on plasma HO-1 level during fever and pyrogenic tolerance in rats

As shown in *Fig. 5* single LPS administration caused an increase in plasma concentration of HO-1 after 4 h (2.03±0.6 ng mL^{-1}) compared to the control group of animals injected with saline (1.22±0.12 ng mL^{-1}) ($p<0.05$). It was, however, lower than in animals injected with CoPP (7.01±0.3 ng mL^{-1}) ($p<0.05$). Three daily injections of LPS resulted in reduction in plasma HO-1 level to 1.17±0.3 ng mL^{-1}. CoPP administrated day before LPS resulted in a prominent increase in the concentration of HO-1 4 h after first (44.51±4.22 ng mL^{-1}) ($p<0.05$) and maintained the elevated level of this protein after third LPS injection (22.28±1.5 ng mL^{-1}) ($p<0.05$).

Figure 5

4. Discussion

Heme oxygenase-1 represents an important research object due to the fact that induction of enzyme activity has significant beneficial or therapeutic effects in a large number of pathologic conditions including endotoxic shock (Fei et al., 2012), ischemia–reperfusion injury (Amersi et al., 1999), rejection of transplanted organs (Öllinger and Pratschke, 2010) and autoimmune neuroinflamation (Chora et al., 2007). Present report clearly demonstrates that heme oxygenase-1 is also a physiological component of the mechanisms that regulates the intensity of the fever and induction of the pyrogenic tolerance to LPS. In presented experiments we used the intraperitoneal injection of cobalt protoporphyrin, a synthetic heme analogue that induces both gene expression (Shan et al., 2006) and the activity of heme oxygenase-1 (Lin et al., 2009). Studies have shown protective effect of HO-1 induced by CoPP in numerous experimental models *in vitro*, and *in vivo* (e.g. Becker et al., 2007; Glanemann et al., 2005; Di Pascoli, 2006). We found that i.p. administration of CoPP (5 mg mL^{-1} i.p.) leads to rise in Tb lasting ca. 8h (*Fig. 1*). The rise in Tb was accompanied by elevation of PGE_2 concentration in plasma (data not showed), and was prevented by an i.p. administration of antibody against HO-1 and COX inhibitor indomethacin. These data indicate that increase in Tb provoked by CoPP, is in fact dependent on HO-1 activation and requires activation of COX (*Fig. 1*). In earlier study we demonstrated that i.c.v. administration of heme L-lysinate, which is a substrate and inducer for HO-1, also provokes a fever-like elevation in Tb and increase in PGE_2 concentration in the cerebro-spinal fluid. Surprisingly, fever provoked by heme appeared to be COX-independent (Walentynowicz et al., 2006). This

fever-like response to heme was, however, inhibited by i.c.v. administration of nonselective HO-1 blocker - zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) (Steiner and Branco, 2000). Taken together, it indicates that induction and activation of HO-1 and further PGE_2 release is a crucial step in the cascade of events leading to rise in Tb after both, CoPP and heme treatment. Different impact of these factors on COX activation remains unclear. It is however known, that free heme possess strong pro-inflammatory properties beside HO-1 induction and is a potent inducer of PGE_2 production in the endothelial cells in a COX-independent way (Haider et al., 2002). CoPP, on the other hand, activates COX to production of prostaglandins by inducing CO release. CO is the only HO-1 product with pyrogenic properties and was shown to affect prostaglandin production via activating COX with or without LPS stimulation (Mancuso et al., 1997; Lin et al., 2010).

Induction of HO-1 protein and activation of its enzymatic activity affected fever induced by i.p. injection of LPS (50µg kg^{-1}) after 24 hours. Pre-injection with CoPP (5 mg kg^{-1}) significantly augmented and enhanced first phase of the febrile response (*Fig. 2*). Obtained results corresponds with earlier observation by Steiner and Branco that inhibition of the enzyme attenuated rise in body temperature evoked by endotoxin (Steiner and Branco, 2001). Furthermore, CoPP augmented IL-6 and PGE_2 levels in plasma after injection of LPS (*Fig. 4*) indicating that HO-1 affects production of endogenous mediators of fever. We also found, that peripheral blood mononuclear cells (PBMC) isolated 24 h after CoPP treatment of rats released increased amounts of IL-6 and PGE_2 after stimulation with LPS *in vitro* (data not showed). Thus, induction of HO-1 and subsequent production of CO may be considered as component of the febrile response that acts above production of cytokines and PGE_2.

The mechanism of pyrogenic tolerance is complex but manifests mainly as reduced ability of immune cells to release cytokines involved in the activation of the febrile processes in the brain (Soszynski et al., 2013; Cavaillon, 1995). In fact, IL-6 production drastically dropped after three consecutive injections of LPS compared to a single injection (*Fig. 4A*). We found that PGE_2 and heme oxygenase-1 levels in plasma also decreased (*Fig. 4B* and *5*) indicating the involvement of these molecules in the molecular mechanism of pyrogenic tolerance. Administration of CoPP resulted in maintaining a high concentration of plasma HO-1 even after three doses of LPS (*Fig. 5*) which led to inhibition of the possibility to achieve pyrogenic tolerance (*Fig. 3*). This was accompanied by elevated levels of PGE_2 but not IL-6 in the plasma (*Fig. 4*). These data are consistent with reports showing that the animals pretreated with heme did not exhibited pyrogenic tolerance to LPS (Raffaini et al., 2006). It can therefore be assumed that the development of tolerance requires a reduction in

the signaling pathway of HO-1. Considering the results presented in this paper the question arises, whether the heme released from damaged tissues in a result of the aseptic injuries (i.e. fracture, tissue injuries) and the subsequent activation of the signaling pathway of HO-1/CO might be a physiological signal breaking the pyrogenic tolerance.

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6. References

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Figures legend

Figure 1. Changes of body temperature (°C) over time (min) of rats treated i.p. at time 0 with CoPP (open circles); CoPP and indomethacin (closed triangles); polyclonal HO-1 antibodies (pAb-HO-1) an hour before (time -60) CoPP (open triangles) and saline as a control (closed circles). Values are means ± S.E.M. at 20-min averages. Letter *n* indicates sample size in each group.

Figure 2. Changes of body temperature of rats treated i.p. with LPS (50 µg kg^{-1}) at time 0, day after i.p. injection with saline (closed triangles) or CoPP (5 mg kg^{-1}) (open circles). Values are means ± S.E.M. at 20-min averages. Letter n indicates sample size in a respective groups.

Figure 3. A) Changes of body temperature of rats pre-injected with saline and treated once (closed triangles), twice (open squares) and three times (open diamonds) with LPS (50 µg kg^{-1} i.p.) at 24 h intervals; **B)** Changes of body temperature of rats pre-injected with CoPP (5 mg kg^{-1} i.p.) and treated once (open circles), twice (closed square) and three times (closed diamonds) with LPS (50 µg kg^{-1} i.p.) at 24 h intervals. Values are means ± S.E.M. at 20-min averages. Letter *n* indicates sample size in a respective groups.

Figure 4. Plasma levels of IL-6 (**panel A**) and PGE2 (**panel B**) 4 h after the first (gray bar) and third (black bar) injection of LPS (50 µg kg^{-1} i.p.) in animals pretreated with saline and 4 h after the first (horizontally hatched lines) and third (vertically hatched lines) injection of LPS in animals pretreated with CoPP (5 mg kg^{-1} i.p.). Values are means ± S.E.M. Letter *n* indicates sample size in a respective groups. Asterisk indicates significant difference (***$p<0.001$).

Figure 5. Plasma levels of HO-1 (ng mL^{-1}) 4 h after first (gray bar) and third (black bar) injection of LPS (50 µg kg^{-1} i.p.) in animals pretreated with saline and 4 h after first (horizontally hatched lines) and third (vertically hatched lines) injection of LPS in animals pre-treated with CoPP (5 mg kg^{-1} i.p.). Chequered bar represents plasma HO-1 concentration in rats 4 h after CoPP injection. Values are means ± S.E.M. Letter *n* indicates sample size in a respective groups. Asterisk indicates significant difference (***$p<0.001$).

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Fig 1

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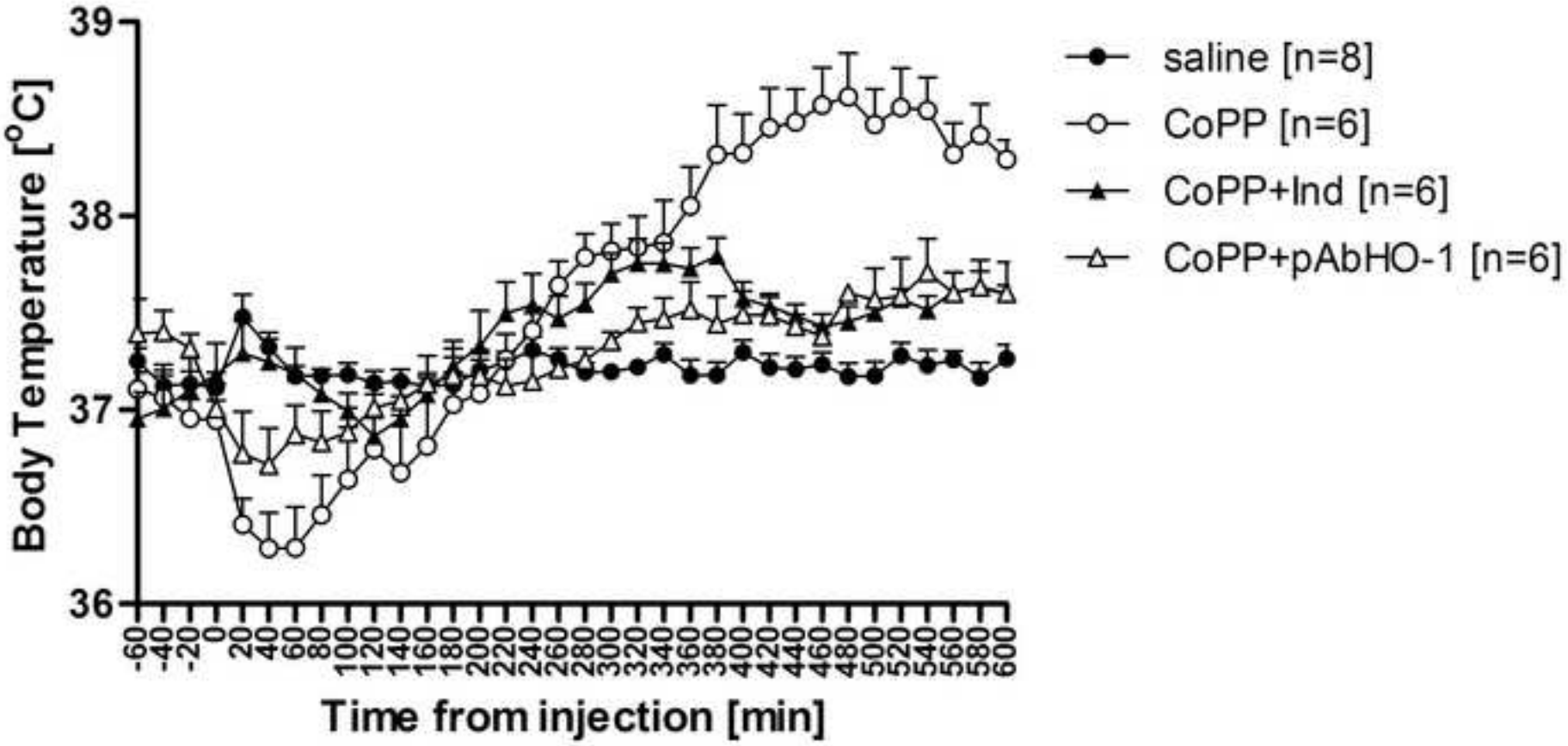

Fig 2

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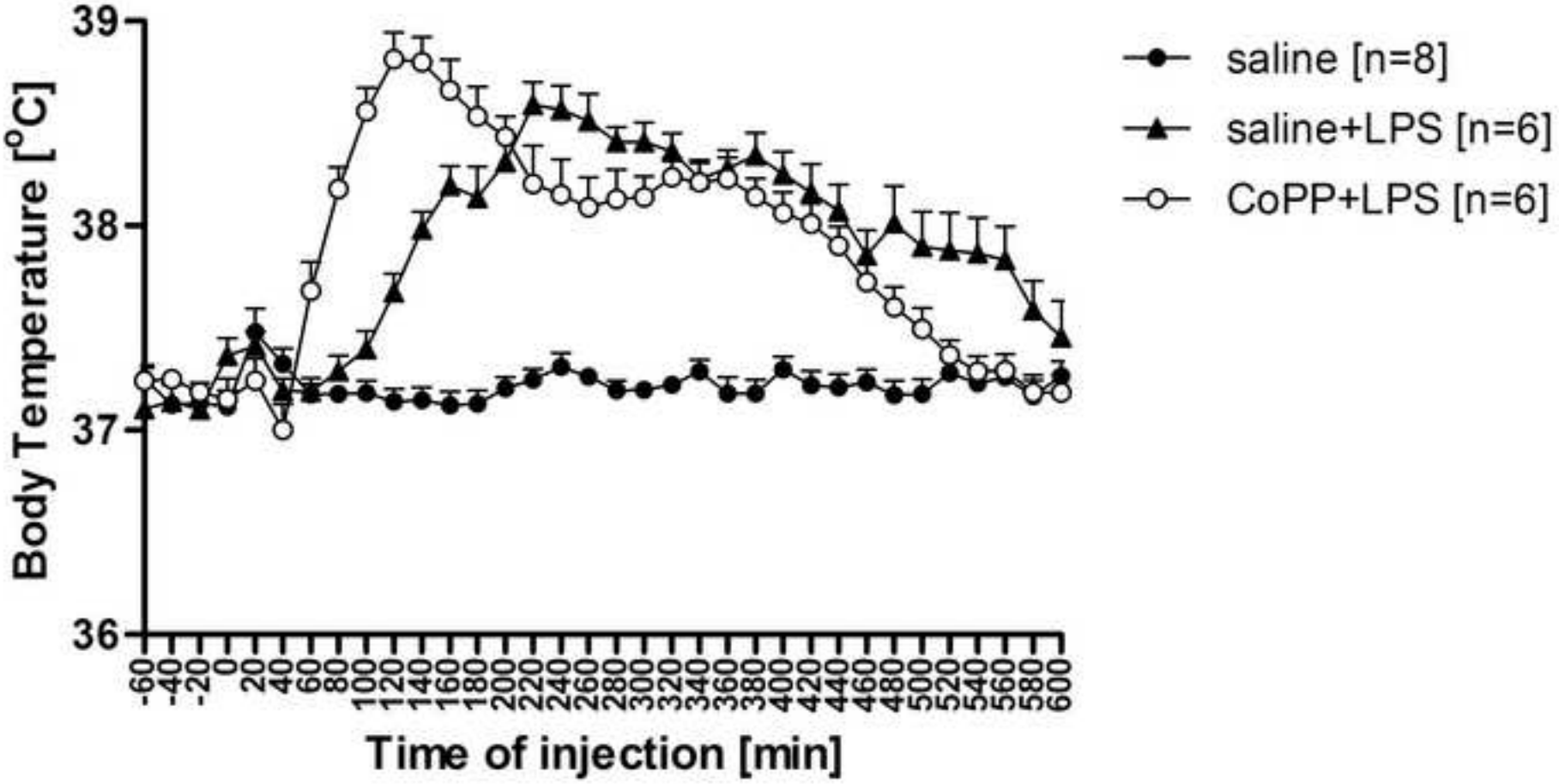

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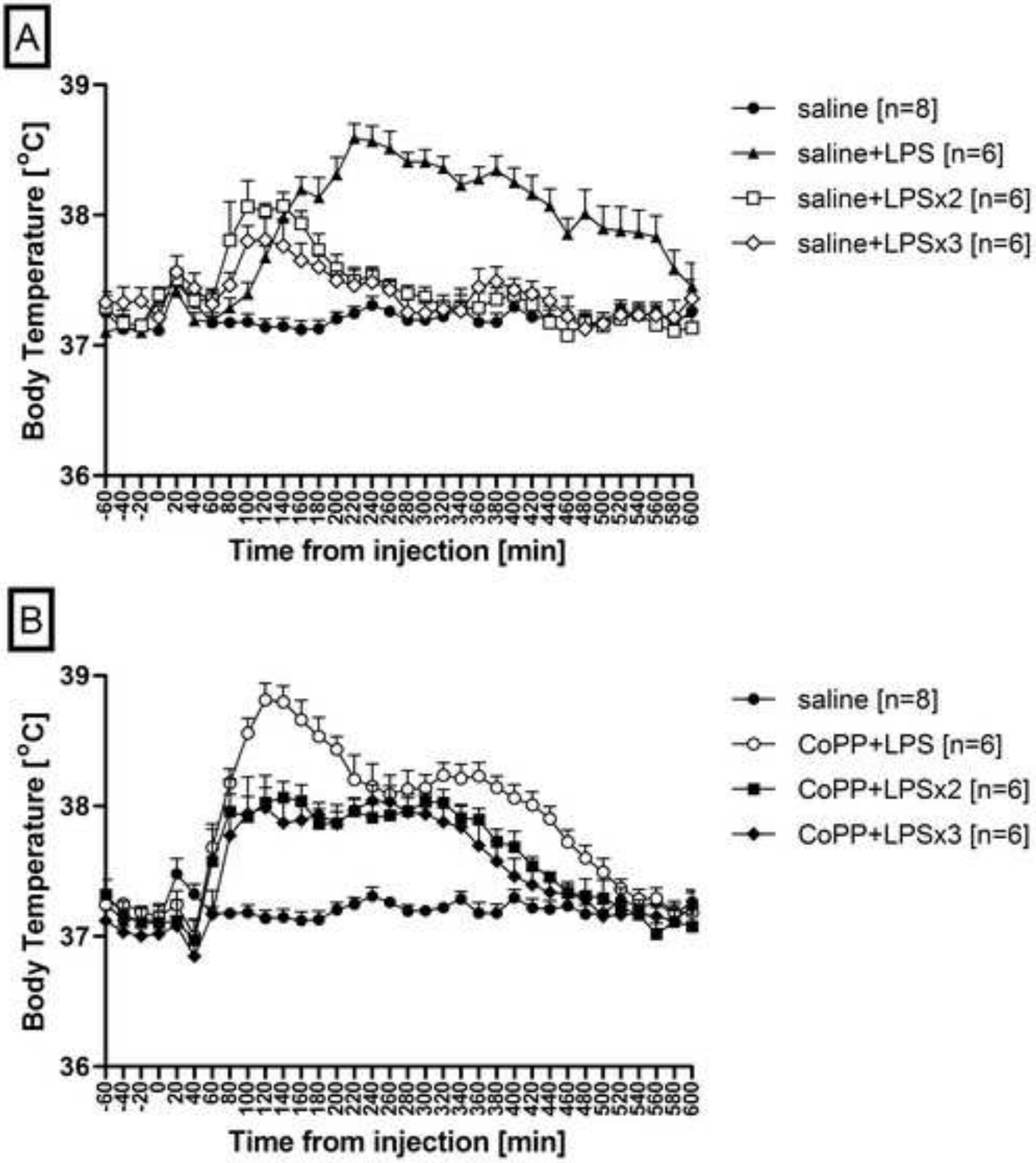

Fig 4

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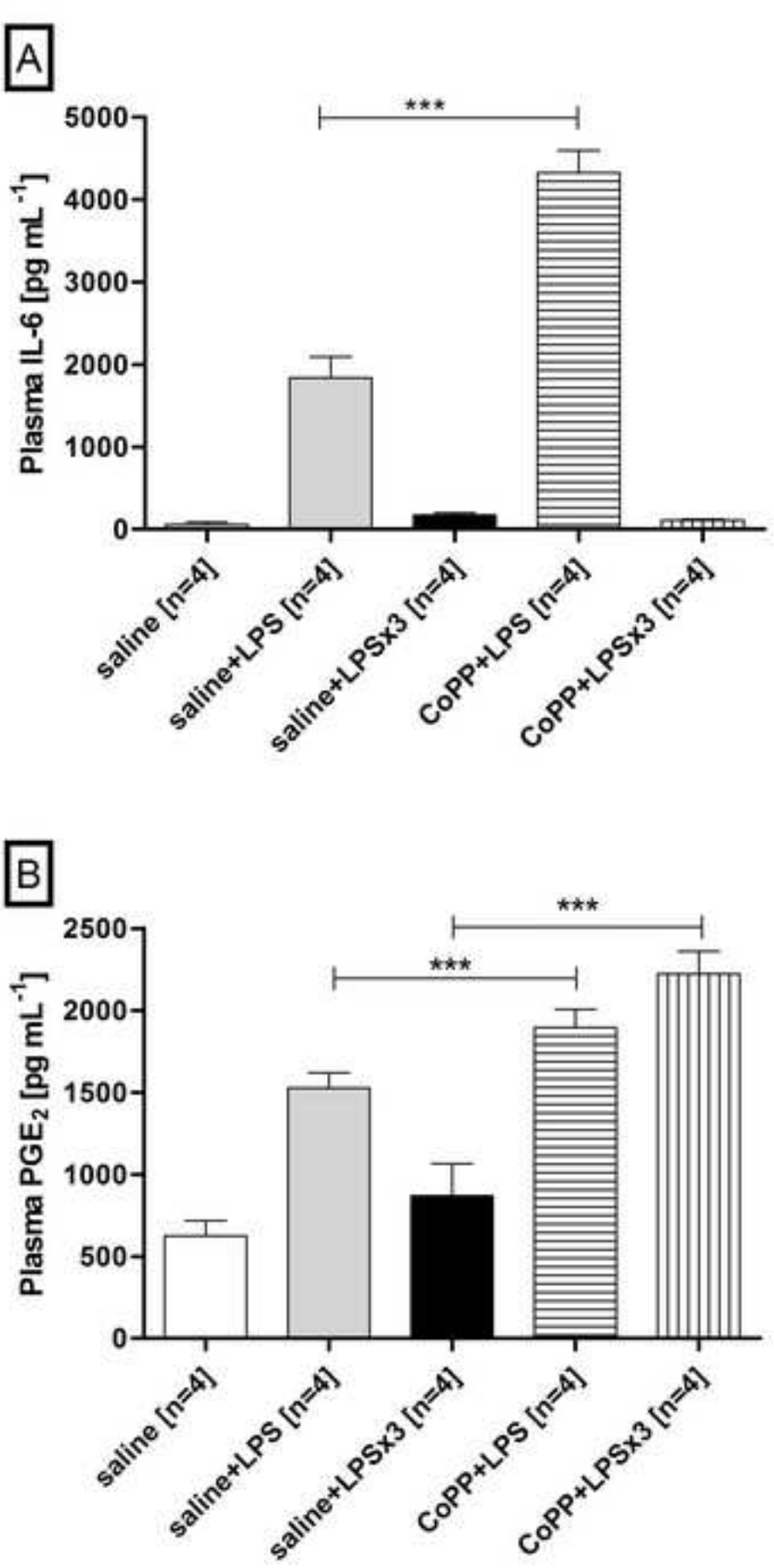

Fig 5

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