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Naltrexone attenuates plasma nitric oxide release following acute heat stress

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Abstract

Previous studies have shown that naltrexone attenuates morbidity and mortality in heat stress by inhibiting endogenous opioids. In this study, we hypothesized that naltrexone can decrease heat stress by attenuating nitric oxide release. Male Sprague–Dawley rats were pretreated with naltrexone or normal saline, and exposed to 45 °C for 25 min; controls were exposed to 25 °C. Colonic temperatures were recorded and plasma samples from an in-dwelling i.v. cannula were analyzed for nitrate/nitrite levels. Following heat stress, peak colonic temperature was significantly diminished ($P<0.05$) in naltrexone-treated rats compared to saline-treated rats. Plasma nitrate/nitrite levels were significantly lower ($P<0.05$) in naltrexone-treated rats compared to saline-treated rats. These findings suggest that naltrexone is able to attenuate the rise in plasma nitric oxide levels commonly observed after heat stress.

Keywords: Hyperthermia; Circulatory failure; Nitric oxide (NO) synthase

1. Introduction

Exposure to high environmental temperatures results in the activation of thermoregulatory centers in the brain and spinal cord. These centers in turn activate appropriate physiological and behavioral responses to maintain the core temperature at the normal level of approximately 37 °C. When these responses are unable to cope with the external heat load, core body temperature rises, leading to possible linical heat stroke (Alzeer et al., 1999). Heat stroke is a systemic disorder characterized by neurological abnormalities (delirium, convulsions, and coma), multiple organ failure with hemorrhage and necrosis in the heart, liver, kidney, and brain, often resulting in death (Simon, 1993).

Naltrexone, an opioid receptor antagonist, is able to attenuate the potentially lethal effects of heat stress. It has been shown to be neuroprotective and capable of reducing both hyperthermic brain injury and core body temperature in rats (Sharma et al., 1997). Animal studies have demonstrated that opioid peptides are released into the blood during heat stress, resulting in hypotension that can be reversed by naltrexone (Holaday, 1983). These findings reinforce the involvement of the endogenous opioid system in the pathophysiology of hyperthermic states (Romanovsky and Blatteis, 1998). It is generally agreed that, in rats, central μ-opioid receptors are likely to be involved in mechanisms that result in heat gain, peripheral κ-opioid receptors regulate heat loss responses, and δ-opioid receptors probably play no part in thermoregulation (Adler and Geller, 1988).

Nitric oxide (NO), which is involved in several physiological processes, such as smooth muscle relaxation, platelet inhibition, neurotransmission, and immune regulation (Moncada et al., 1991), has been shown to play a role in the pathophysiology of heat stroke (Bouchama et al., 1991; Hall et al., 1994) as well. A 70% increase in NO production was observed in both the cerebellum and the cortex of saline-treated rats during heat stress (Canini et al., 1997). It has been suggested that during conditions of heat stress, a selective loss of compensatory splanchnic vasoconstriction may lead to circulatory failure (Kregel et al., 1988) and trigger the cascade of events that characterize heat stroke (Morimoto et al., 1998). Enhanced local release of NO within the splanchnic circulation of rats has been shown

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to precede this loss of splanchnic vasoconstriction (Hall et al., 1994). Elevated levels of NO observed in the circulation of heat stroke patients (Alzeer et al., 1999) further reaffirms the role of NO in the development of heat stroke.

Previous studies have associated the beneficial effects of naltrexone in heat stress to its antagonism of opioid peptides (Sharma et al., 1997), which are thought to play a role in central nervous system cell injury (Faden, 1990). So far, studies have not linked the use of naltrexone to NO levels in heat stress. In this study, we focused on our hypothesis: that naltrexone attenuated morbidity and mortality by reducing NO release during conditions of heat stress. We developed a heat stress protocol that delivered an acute heat stress with the rapid induction of heat stroke. Naltrexone was administered i.v. to Sprague–Dawley rats, which were then restrained and subjected to total body hyperthermia, using a heat stress protocol of exposure to a temperature of 45 °C for 25 min, with relative humidity 55%, in a climatic chamber.

2. Materials and methods

2.1. Animals

The animals were handled in accordance with the guidelines of the Council for International Organization of Medical Sciences (CIOMS) ethical code for animal experimentation (Howard-Jones, 1995). Male Sprague–Dawley rats (250–300 g) were used in the study. The rats were housed in a temperature-controlled animal facility with a 12-h light–dark cycle and were provided standard rat chow and water ad libitum. Rats were anesthetized with Clinical Research Center (CRC) cocktail (i.p. 0.33 ml 100/g), containing 1 part Hypnorm (Jansen Pharmaceutica, Beerse, Belgium), which contains fentanyl (0.315 mg/ml) and fluanisone (10 mg/ml), 1 part Dormicum (Roche, Basel Switzerland), which contains midazolam (5 mg/ml), and 2 parts water for injection, before being cannulated in the right femoral vein. Cannulas were made of silicone rubber tubing; PE 50 and PE 10 polyethylene tubing (Intramedic, Clay Adams) joined using Araldite (Ciba Specialty Chemicals). Heparinized (Heparin Sodium Injection, B. Braun) normal saline (100 IU/ml) was used to flush the cannulas. After cannulation, the cannulas were threaded under the skin dorsally and were made to exit at the dorsal nape of the neck, just above the shoulder blades. The cannulas were held in place by means of dental cement (Harry J. Bosworth) and capped with small steel pins. Baneocin (250 IU/ bacitracin zinc. B.P., 5000 IU neomycin, as sulphate B.P., Biochemie GmbH, Vienna, Austria) was applied to all surgical sites after the incisions were closed with sutures. The rats were allowed to recover for 48 h prior to being subjected to heat stress. The implanted cannulas were used for drug administration and for drawing blood samples at designated intervals.

Four groups of animals were used in the study: (i) naltrexone-treated ($n=5$) and (ii) saline-treated rats subjected to heat stress ($n=5$), and (iii) control naltrexone-treated ($n=5$) and (iv) saline-treated ($n=5$) rats without heat stress. The doses given were naltrexone (Sigma) 10 mg/kg, or saline 0.9% 1 ml/kg, via the cannulated femoral vein. The dose of naltrexone was selected based on published data (Sharma et al., 1997), which demonstrated a positive response to heat stress with the selected drug dose. Naltrexone was dissolved in normal saline to obtain 10 mg/ml solutions. All drugs were administered 1 h prior to the start of experiments.

2.2. Heat stress protocol

The cannulated rats were familiarized with the testing environment 24 h before the experiments were performed. All experiments were conducted between 2 p.m. and 6 p.m. to minimize variations associated with the animals' circadian rhythms. The rats were restrained in custom-made Perspex restrainers. Thermistor probes were inserted about 5 cm into the rectum of each animal. A six-channel thermistor thermometer (Cole Parmer) was used to read off colonic temperatures. Animals were exposed to 45 °C heat stress, relative humidity 55% for 25 min in a climatic chamber (Cold–Heat-Climate-Test chamber, Weiss Technik). Control animals were exposed to identical conditions as the heat stressed animals, but at 25 °C. Blood samples were taken before, immediately after and 1 h after heat stress ($t=0$, 25, and $t=60$ min). Blood volumes of 0.6 ml were drawn each time and were replaced with an equal volume of normal saline (0.9%). Blood samples were centrifuged at $3000 \times g$ for 10 min to obtain platelet-rich plasma, which was than assayed for NO, using an enzyme-linked immunosorbent assay (ELISA). Nitrate/Nitrite colorimetric assay kits (Cayman Chemical) were used to estimate NO. Plasma NO was calculated from plasma nitrate/nitrite levels based on the Griess reaction.

2.3. Statistical analysis

Differences between the treatment groups were assessed by analysis of variance (ANOVA), followed by post hoc analysis of the data (Bonferroni), using SPSS version 10. Paired *t*-tests were performed for the within group analysis, where nitrate/nitrite levels were analyzed before and after heat stress.

3. Results

3.1. Nitric oxide

Fig. 1 shows the plasma nitrate/nitrite levels at $t=0$, 25, and 60 min for both the control and heat stress groups. Naltrexone-treated control rats showed no statistically sig-

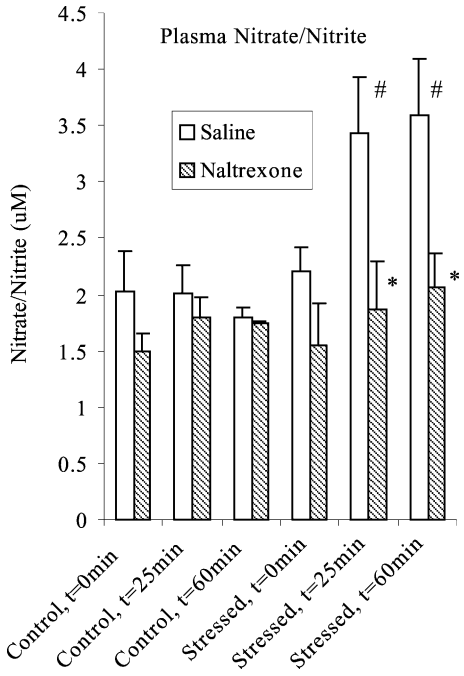

Fig. 1. Time plots of plasma nitrate/nitrite levels in control and heat-stressed groups. Each point is the mean ± S.E.M. (*: $P<0.05$ compared to saline-treated, heat-stressed rats; #: $P<0.05$ compared to the respective saline and naltrexone-treated, heat-stressed rats at $t=0$ min).

nificant differences in the nitrate/nitrite levels at all three time points, compared with the saline-treated control rats. In the heat stress groups, only the saline-treated rats displayed significant elevations in plasma nitrate/nitrite levels ($P>0.05$) at $t=25$ and 60 min, compared to levels prior to stress at $t=0$ min. The high plasma nitrate/nitrite levels in the heat-stressed, saline-treated rats, were significantly

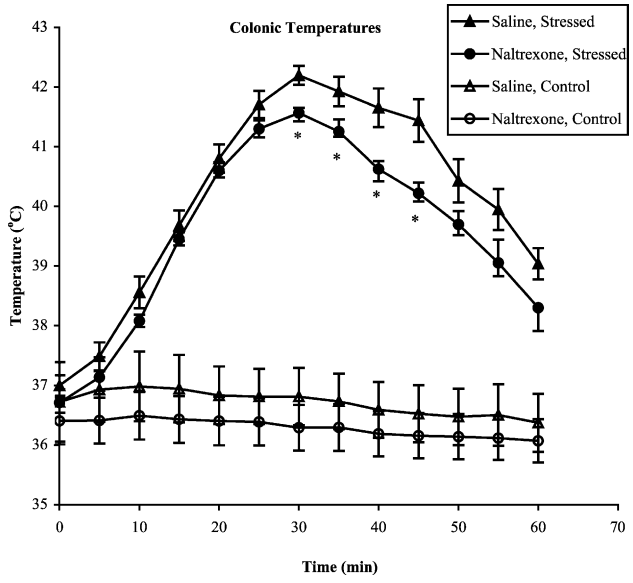

Fig. 2. Colonic temperature time plots of control and heat-stressed groups. Each point is the mean ± S.E.M. (*: $P<0.05$ compared to saline-treated, heat-stressed rats).

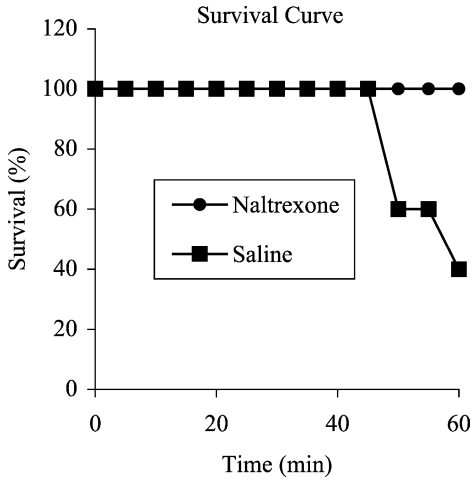

Fig. 3. Time plots of survival of the heat-stressed groups.

greater ($P<0.05$) than the corresponding levels in naltrexone-treated rats at $t=25$ and 60 min. Following heat stress, no statistically significant differences in plasma nitrate/nitrite were observed in the naltrexone-treated rats. The plasma nitrate/nitrite levels of both the control and heat-stressed, naltrexone-treated rats were similar to each other and not statistically significantly different.

3.2. Colonic temperature

Fig. 2 shows the colonic temperatures of both the control and heat-stressed groups. Control rats maintained steady colonic temperatures from $t=0$ to $t=60$ min, showing statistically insignificant differences. Under conditions of heat stress, peak colonic temperature was reached at $t=30$ min for both groups. Heat-stressed, saline-treated rats attained a maximum colonic temperature of 42.19 ± 0.16 °C, while the naltrexone-treated rats reached 41.56 ± 0.14 °C ($P<0.05$, compared with the saline-treated rats). Significantly lower colonic temperatures ($P<0.05$) in naltrexone-treated rats were also observed at time points $t=35$, 40, and 45 min, compared with the saline-treated rats.

3.3. Survival curve

Fig. 3 shows the survival rate of the heat-stressed groups. The survival rate was 100% for the control groups. Under heat stress, the naltrexone-treated rats showed a 100% survival from $t=0$ to $t=60$ min. The saline-treated rats showed a 60% survival (3/5) at $t=50$ min. This fell further to 40% (2/5) at $t=60$ min.

4. Discussion

Heat stress was achieved by exposing the animals to an elevated environmental temperature of 45 °C for 25 min in a climatic chamber, while control animals were exposed to a

temperature of 25 °C for a similar duration. We developed a heat stress protocol to achieve a colonic temperature of 41.5 °C or higher, a temperature widely accepted as the temperature marker for heat stress (Hall et al., 2000; Kregel and Moseley, 1996). Exposure to 45 °C for 25 min caused the rectal temperature to rise rapidly to values higher than 41.5 °C. Several animal studies have shown that heat stroke develops following a drastic drop in mean arterial blood pressure (Kregel et al., 1988; Lin, 1999) These studies also indicate that mean arterial blood pressure falls rapidly when the rectal temperature exceeds 41.5 °C.

Naltrexone was used in this study to investigate its effects on NO levels before and after exposure to heat stress. Significant elevations in plasma nitrate/nitrite levels were observed in saline-treated rats after heat stress (Fig. 1). The use of naltrexone attenuated this elevation of NO. There are several explanations for the elevation of NO levels seen after exposure to heat stress. It has been reported that an increase in the release of cytokines, such as tumor necrosis factor and interleukin-1, occurs in heat-stressed patients and rats (Bouchama et al., 1991; Lin et al., 1997). These cytokines can up-regulate the inducible form of nitric oxide synthase (iNOS) found predominantly in macrophages, resulting in the enhanced production of NO from L-arginine (Moncada et al., 1991). Furthermore, during conditions of heat stress, endothelial activation was observed and this supports the role of NO as a possible mediator in heat stress (Bouchama et al., 1996). Elevated NO levels were also observed in the splanchnic circulation of rats after heat stress (Kregel et al., 1988) and in hyperkinetic states such as sepsis and burns (Preiser et al., 1996; Kilbourn and Griffith, 1992; Jeschke et al., 2001), and these levels have been associated with circulatory collapse. It was recently reported that NO did not contribute to the hypotension of heat stroke (Ryan et al., 2001). However, this finding is not relevant to our study, as it was based on ketamine-anesthetized rats, while our study is based on conscious rats.

A relationship between endogenous opioids and NO was proposed when it was shown that iNOS activation, following lipopolysaccharide injection, was mediated by endogenous opioids and was reversed by naltrexone in rats (Lysle and How, 1998). It is thus possible that naltrexone could have inhibited endogenous opioids that were responsible for the activation of iNOS. Another possibility is that the increased levels of endogenous opioids observed during heat stress could have increased endothelial intracellular calcium, thereby increasing NO release by constitutive nitric oxide synthase (cNOS)(Way et al., 1998). Hence, it is possible that in our study, naltrexone could have inhibited the action of endogenous opioids, thus attenuating NO release.

Several studies have demonstrated the inhibition of iNOS by glucocorticoids (Drew and Chavis, 2000; Moncada et al., 1991; Palmer et al., 1992), thereby inhibiting NO production. It is established that naltrexone can increase cortisol and corticosterone release in rats via an increase in adrenocorticotropic hormone (Tsagarakis, 1992). A link between cortisol and NO was further proposed when cortisol was shown to decrease NO release, potentially through the inhibition of transmembrane arginine transport and the inhibition of iNOS in rat tissues (Simmons et al., 1996). Cortisol administration was also shown to reduce plasma nitrate/nitrite levels in humans (Kelly et al., 1998). It has been recently reported (Hall et al., 2001) that iNOS activation contributes to hyperthermia-related splanchnic dilation. Thus, besides the inhibition of endogenous opioids, naltrexone could have enhanced cortisol and corticosterone release under conditions of heat stress, and this elevated level of glucocorticoids could have in turn reduced the activation of NOS. An analysis of plasma cortisol and corticosterone levels with naltrexone treatment and heat stress would provide more insight into this theory.

As expected, in our study, naltrexone-treated rats attained lower maximum colonic temperatures (Fig. 2) than did saline-treated rats (Sharma et al., 1997). Injection of naltrexone into the pre-optic anterior hypothalamus of rats attenuated the hyperthermia induced by handling stress (Pae et al., 1985), suggesting that the endogenous opioid system plays a role in hyperthermia. Our findings indicate a significant reduction in the maximum colonic temperature reached by the naltrexone-treated rats, compared to the saline-treated rats, under conditions of heat stress. A potential site for the antagonistic action of naltrexone is the μ-opioid receptors, which are widely believed to be involved in heat gain mechanisms in rats (Adler and Geller, 1988).

This study demonstrated the ability of naltrexone to attenuate mortality from heat stroke. All naltrexone-treated rats survived compared to 40% of the saline-treated rats (Fig. 3). A probable cause of the observed mortality could be circulatory failure, preceded by the loss of splanchnic vasoconstriction (Kregel et al., 1988; Morimoto et al., 1998), as observed under conditions of hyperthermia in animal studies. The physiological response to heat stress in animals includes cutaneous vasodilation, to transfer heat from the core of the body to the body surface. This leads to the lowering of the central venous pressure and dehydration from evaporative heat loss. The cardiovascular system responds to heat stress by maintaining cardiac output in response to an increased demand for skin blood flow. At moderate hyperthermia (~ 40 °C), mean systemic arterial pressure is maintained with an increase in cardiac output, because of an increase in heart rate and stroke volume. Central venous pressure is maintained, with a linear decrease in total peripheral vascular resistance, as the core temperature rises. As body temperature rises to ~ 42 °C (severe hyperthermia), central venous pressure falls as blood shifts from the central circulation to the systemic circulation, resulting in circulatory failure (Miki et al., 1983).

In conclusion, we have demonstrated that naltrexone is able to minimize mortality due to heat stress, potentially by attenuating NO release. This attenuation is likely to be mediated by the naltrexone-induced inhibition of endogenous opioids, which are released during heat stress.

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