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**ROLE OF THE NITRIC OXIDE SYNTHASE ISOFORMS DURING MORPHINE-
INDUCED HYPERTHERMIA IN RATS**

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Running title: NOS isoforms and morphine hyperthermia

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Abbreviation: 7-NI, 7-nitroindazole; L-NIO, N(5)-(-iminoethyl)-L-ornithine; AG, aminoguanidine AG; L-NAME, N-nitro-L-arginine methyl ester; NO, nitric oxide; Tb, body temperature; POAH, preoptic anterior hypothalamus. CNS, central nervous system.

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Abstract

Recently, we demonstrated that the diffusible messenger molecule nitric oxide (NO) is involved in the hyperthermic response induced by morphine by using a non-selective nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME). The present work extended these studies to include 7-nitroindazole (7-IN), an inhibitor specific for neuronal nitric oxide synthase (nNOS), N(5)-(-iminoethyl)-L-ornithine (L-NIO), an inhibitor of endothelial nitric oxide synthase NOS (eNOS), and aminoguanidine (AG), a potent inhibitor of inducible nitric oxide synthase (iNOS). A biotelemetry system was used in this study to measure the body temperature (Tb). A dose of 7-NI (5 or 10 mg/kg) that did not affect Tb by itself, blocked, in a dose-dependent manner, the hyperthermia induced by morphine (15 mg/kg i.p.). However, pretreatment with L-NIO (10-20 mg/kg) or with AG (50 mg/kg) failed to alter the hyperthermia induced by morphine. L-NIO (10-20 mg/kg) or AG (50 mg/kg), per se, had no effect on Tb. These results suggest the involvement of nNOS in morphine-induced hyperthermia.

Morphine has effects on a number of physiological functions, including antinociception, respiration, gastrointestinal function, blood pressure, immunoregulation and thermoregulation. With respect to the last, previous results from this and other laboratories demonstrated that intracerebroventricular (i.c.v.) administration of selective *mu* receptor agonists produced hyperthermia (Adler and Geller, 1993; Handler et al., 1992; Xin et al., 1997) that could be blocked or antagonized by selective *mu* receptor antagonists, whereas *kappa* receptor agonists produced hypothermia (Xin et al., 1997) that could be blocked or antagonized by selective *kappa* receptor antagonists (Handler et al., 1992). These findings indicate that the hyperthermic response to opioids in rats is mediated by the *mu* receptor and the hypothermic response is mediated by the *kappa* receptor (Xin et al., 1997). The thermoregulatory effects of *delta*-opioid receptors are not as clear. While some studies observed hypothermia after the application of *delta*-receptor agonists (Spencer et al 1988), others did not find a significant change in body temperature (T_b) (Handler et al 1992).

In addition, several studies strongly support the involvement of the opioid system in the fever process. Thus, *mu* opioid-receptor-selective antagonists given into the preoptic anterior hypothalamus (POAH) block the fever induced by lipopolysaccharide (Benamar et al., 2000), interleukin-6 (Benamar et al., 2002). Nitric oxide (NO), recently recognized as a prominent second messenger (Breder and Saper, 1996), is produced by the enzyme nitric oxide synthase (NOS) that utilizes L-arginine to make L-citrulline and the radical gas NO. NOS has been found in peripheral and central neurons (Bredt and Snyder, 1992). Three different isoforms of NOS have been described (Lopez-Figueroa et

al., 1998). Two are constitutive forms, endothelial and neuronal (Moncada et al., 1991), and the third is inducible (Lowenstein et al., 1992). nNOS was first described in the neurons of the central and peripheral nervous systems. eNOS is generally found in the endothelium of blood vessels responsible for vasodilatation. iNOS is located in the cytosol of cells in the immune system, in smooth muscles and hepatocytes. It was originally described as inducible and is almost undetectable under basal conditions but is induced at the transcriptional level by LPS and cytokines (Xie et al., 1992).

Within the last few years, a number of studies have been conducted to investigate whether NO plays a role in temperature regulation, fever and hypothermia. Some authors have suggested that NO has an antipyretic function (Gourine, 1995; Moncada et al., 1991), and some have shown that NO is involved in hypothermia (Almeida and Branco, 2001; Branco et al., 1997; Steiner et al., 1998). However, other reports provide evidence that the formation of NO participates in the development of a febrile response (Benamar et al., 2000; Lin and Lin, 1996; Roth et al., 1998; Scammell et al., 1996). It should be noted that these studies differed in strategies to assess the role of NO, species of experimental animal, pyrogen administered and route of administration, as well as in inhibitors used.

Although NO has been implicated in the hyperthermia induced by morphine (Benamar et al., 2001), the NOS isoform involved in the induction of NO remains unknown. Therefore, the present study was undertaken to identify which type of NOS isoform is involved in morphine-induced hyperthermia, using selective inhibitors of the 3 types of NOS isoforms.

Material and methods

Animals

All animal use procedures were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use committee. Male Sprague-Dawley rats (Zivic-Miller) weighing 250-300 g were used in this study. They were housed two per cage for at least one week before surgery and were fed laboratory chow and water ad libitum. The ambient temperature was 22 ± 2 °C and 12 h light/12 h dark cycle was used. All experiments were started between 09: 00 and 10: 00 h to minimize the effect of circadian variation in Tb.

Surgery procedures

Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (100-150 mg/kg) and acepromazine maleate (0.2 mg/kg). An incision 2 cm in length was made along the linea alba, and the underlying tissue was dissected and retracted. A transmitter (Mini-Mitter, Sunriver, OR) was implanted in the intraperitoneal space. After the transmitter was passed through the incision, the abdominal musculature and dermis were sutured independently. The animals were returned to individual cages in the environmental room.

Measurement of body temperature

One week after surgery, the rats were tested in an environmental room (21 ± 0.3 °C ambient temperature and 52 ± 2 % relative humidity). Tb was measured by a biotelemetry system (Mini-Mitter, Sunriver, OR) using calibrated transmitters implanted

i.p. Signals from the transmitter were delivered through a computer-linked receiver. This method minimizes stress to animals during the Tb reading. Thus, the Tb could be monitored continuously and recorded without restraint or any disturbance to the animal.

Drugs

Morphine sulfate (National Institute on Drug Abuse) was dissolved in sterile pyrogen-free saline. AG was purchased from Sigma (St Louis, MO) and was dissolved in saline. L-NIO and 7-NI were obtained from Sigma (St Louis, MO), and were dissolved in DMSO.

Statistical analysis

All results were expressed as mean \pm S.E.M. Statistical analysis of differences between groups was determined by analysis of variance (ANOVA) followed by Tukey's post hoc test. A value of P less than 0.05 was considered statistically significant.

Results

Effect of i.p. injection of 7-NI on body temperature

During the 180-min recording period, no significant change in Tb was observed after injection of 7-NI at doses of 5 or 10 mg/kg i.p., as compared to the effect of injection of an equivalent volume of vehicle (DMSO) (Fig.1, $P > 0.05$). However, 7-NI at dose of 20 mg/kg i.p. altered Tb significantly as compared to the effect of injection of an equivalent volume of vehicle (DMSO) (Fig.1, $P < 0.05$). Accordingly, a dose of 5 or 10 mg/kg was used in these experiments to investigate the role of nNOS during morphine hyperthermia. Mean Tb prior to injection was 37.56 ± 0.14 for the 5 mg/kg 7-NI group, 37.63 ± 0.17 for the 10 mg/kg group and 38.10 ± 0.18 for 20 mg/kg group.

Effect of i.p. injection of 7-NI on morphine-induced hyperthermia

7-NI, injected 30 min before morphine blocked the morphine-induced hyperthermia (Fig. 2). The effect of 7-NI was dose-dependent. Doses of 5 or 10 significantly suppressed the morphine-induced hyperthermia (Fig. 2, $P < 0.05$). Mean Tb before injection was 37.79 ± 0.05 for the saline/morphine group, 37.66 ± 0.17 for the 7-NI (20 mg/kg)/Morphine group, 37.60 ± 0.17 for the 7-NI (10mg/kg)/morphine group and 37.87 ± 0.23 for the 7-NI (5 mg/kg)/morphine group.

Effect of i.p. injection of AG on morphine-induced hyperthermia

Pretreatment with AG (20 mg/kg) failed to alter the hyperthermic response during the 180-min recording period (Fig. 3, $P > 0.05$). No significant change in Tb was observed after AG injection compared to the effect of injection of an equivalent volume of vehicle (saline) (Fig.3, $P > 0.05$). Mean Tb before injection was 37.42 ± 0.14 for the saline/morphine group, 37.49 ± 0.12 for the AG/morphine group and 37.44 ± 0.15 for the AG group.

Effect of i.p. injection of L-NIO on hyperthermia induced by morphine

To determine whether eNOS mediated the morphine hyperthermia, L-NIO was injected i.p. 30 min before morphine. L-NIO (10 mg/kg) did not alter the morphine hyperthermia (Fig.4, $P > 0.05$). Injection of L-NIO alone did not affect Tb (Fig.4, $P > 0.05$). Mean Tb before injection was 37.82 ± 0.10 for the saline/morphine group, 37.77 ± 0.12 for the L-NIO/morphine group and 37.88 ± 0.14 for the L-NIO group.

Discussion

The acute administration of morphine at doses ranging from 4 to 15 mg/kg produces hyperthermia in rats (Benamar et al., 2001; Geller et al., 1982). Similarly, in the present study, the injection of 15 mg/kg induced hyperthermia in rats. The NO synthase and the three types of opioid receptors, *mu*, *kappa* and *delta* are widely distributed in the CNS. Dense NOS staining occurs in the hypothalamus (Bredt and Snyder, 1992) and opioid receptors are also present in the hypothalamus, including in the preoptic anterior hypothalamus, a vital region in Tb (Mansour et al., 1987). It has been demonstrated that NO plays an important modulator role not only in the cardiovascular system but also in other physiological and pathophysiological processes, including thermoregulation, fever and hyperthermia (Amir et al., 1991; Gerstberger, 1999; Steiner et al., 2002). With respect to the last, we recently demonstrated that blocking the endogenous NO production by systemic injection of L-NAME (a non-selective NOS inhibitor) suppressed the hyperthermic response induced by morphine 15 mg/kg (Benamar et al., 2001), indicating a clear involvement of NO during morphine-induced hyperthermia. Although L-NAME caused changes in the hyperthermic response induced by morphine, it is a non-selective NOS inhibitor and acts on both constitutive and inducible isoforms of the enzyme. In order to identify the NOS isoforms involved in morphine hyperthermia, we used nNOS, iNOS and eNOS inhibitors. Pharmacokinetic studies show that the 7-NI inhibits rat cerebellar NOS activity in vivo and in vitro in different brain regions, with maximum inhibition occurring 30 min after i.p. administration and returning to normal slowly in 24 h (MacKenzie et al., 1994). Under the present experimental conditions, i.p. injection of 7-

NI at doses of 5 or 10 mg/kg itself caused no modification in Tb. However, 7-NI blocked the hyperthermia induced by morphine (15 mg/kg) in a dose-dependent manner, with maximum inhibition occurring at 30 min, coinciding with the pharmacokinetics of 7NI (MacKenzie et al., 1994). Our results thus demonstrate that nNOS participates in the hyperthermic effect of morphine and are compatible with the hypothesis that nNOS is involved in the mediation of other types of hyperthermia. Indeed, the hyperthermia caused by heat stress is associated with marked upregulation of nNOS in many parts of the brain (Sharma et al., 1999). In addition, Western blotting identified nNOS in homogenates of whole brain, and specific immuno-fluorescence staining showed a high nNOS signal in the POAH during LPS-induced fever in rats (Gath et al., 1999). In addition, i.p. administration of 7-IN at a dose of 25 mg/kg significantly attenuated the rise in Tb elicited by restraint stress, indicating that the nNOS isoform plays an important role in the development of restraint stress-induced fever (Sanches et al., 2002). Similarly, pretreatment with 7-IN blocked the fever induced by LPS (Perotti et al., 1999).

Morphine is routinely used for pain management in the critically ill, it is thus of clinical relevance to determine the role of NO as a mediator of physiological and pathological process inn the hyperthermic response induced by morphine. Recently we have demonstrated that L-NAME blocks the hyperthermia induced by morphine (Benamar et al., 2001). However, L-NAME produces an increase in blood pressure. In the present study we could prevent the morphine-induced hyperthermia by using 7-NI, which did not increase blood pressure (Mackenzie et al., 1994).

In order to investigate the role of iNOS in the hyperthermia induced by morphine, we used AG. Pretreatment with a dose of AG (50 mg/kg) that did not affect the Tb did not alter the hyperthermia induced by morphine. Interestingly, a similar effect of AG (50 mg/kg) has been found in restraint stress-induced fever in rats (Sanches et al., 2002) and in LPS-induced fever in pigs (Roth et al., 1999). Also, it has been demonstrated that inducible-type iNOS could neither be detected by immunofluorescence staining nor on Western blots in preparations from rats treated with a pyrogenic dose of LPS (Gath et al., 1999). Moreover, a very small population of iNOS-positive microglial cells appeared several hours after administration of LPS in rats (VanDam et al., 1995), and no significant elevation of NO activity is detected in brain a short time after LPS administration (Sehic et al., 1997). The iNOS is almost undetectable under basal conditions but is induced at the transcriptional level by LPS and cytokines (Xie et al., 1992) until the enzyme is synthesized, a process that requires several hours (Mustafa and Olson, 1998). The administration of morphine induced an increase in Tb starting 10-20 min after injection, before iNOS was produced. That AG failed to alter the hyperthermia induced by morphine seems reasonable. However, some authors have shown that iNOS is detected in hypothalamic cells (Miñano et al., 1997) after LPS administration, and intra-OVLT (organum vasculosum of the lamina terminalis) injection of AG inhibited the fever induced by LPS (Lin and Lin, 1996). It should be noted that these studies varied in conditions used to assess the role of NO.

The implication of eNOS as the source of NO whose production was stimulated by LPS has reported recently (Yang and Krukoff, 2000). Also, glial gene expression of

eNOS was shown to increase in response to viral infection (Barna et al., 1996) suggesting that, under special circumstances, glia can be induced to produce greater amounts of eNOS. In this study, the possible participation of eNOS has been investigated by using L-NIO, an inhibitor that acts predominantly on eNOS but also has effects on nNOS. Neither 10 mg/kg nor 20 mg/kg of L-NIO by itself altered Tb. The pretreatment with L-NIO did not affect morphine-induced hyperthermia, suggesting that eNOS does not play a role in mediating the hyperthermic response to morphine.

In conclusion, the present studies have demonstrated that morphine, injected at dose of 15 mg/kg i.p. in rats, produced hyperthermia, and the effect could be blocked by an nNOS inhibitor but not by iNOS nor by nNOS inhibitor, underscoring the differing roles of NOS isoforms in the hyperthermic processes. This finding indicates clearly that NO produced by nNOS mediates morphine-induced hyperthermia. However, eNOS and iNOS seem not to be involved in the morphine-induced hyperthermia

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Footnotes

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Figure legends

Fig.1. Effect of i.p. administration of 7-NI (5, 10 and 20 mg/kg) on Tb. 7NI was injected at time zero. Data are expressed as the mean \pm S.E.M. from baseline. N, number of rats. Δ Tb, variation in body temperature. * P< 0.05

Fig.2. Effect of 7-NI on morphine-induced hyperthermia (15 mg/kg, i.p.). 7-NI was injected 30 min prior to morphine. Data are expressed as the mean \pm S.E.M. from baseline. N, number of rats. Δ Tb, variation in body temperature. * P< 0.05

Fig.3. Lack effect of AG (50 mg/kg, i.p.) does not alter the morphine-induced hyperthermia (15 mg/kg, i.p.). Morphine was injected at time 0 min. AG was injected 30 min before. Data are expressed as the mean \pm S.E.M. from baseline. N, number of rats. Δ Tb, variation in body temperature.

Fig.4. Effect of L-NIO on morphine-induced hyperthermia (15 mg/kg, i.p.). Morphine was injected at time 0 min. L-NIO was injected 30 min before. Data are expressed as the mean \pm S.E.M. from baseline. N, number of rats. Δ Tb, variation in body temperature.

FIGURE 1

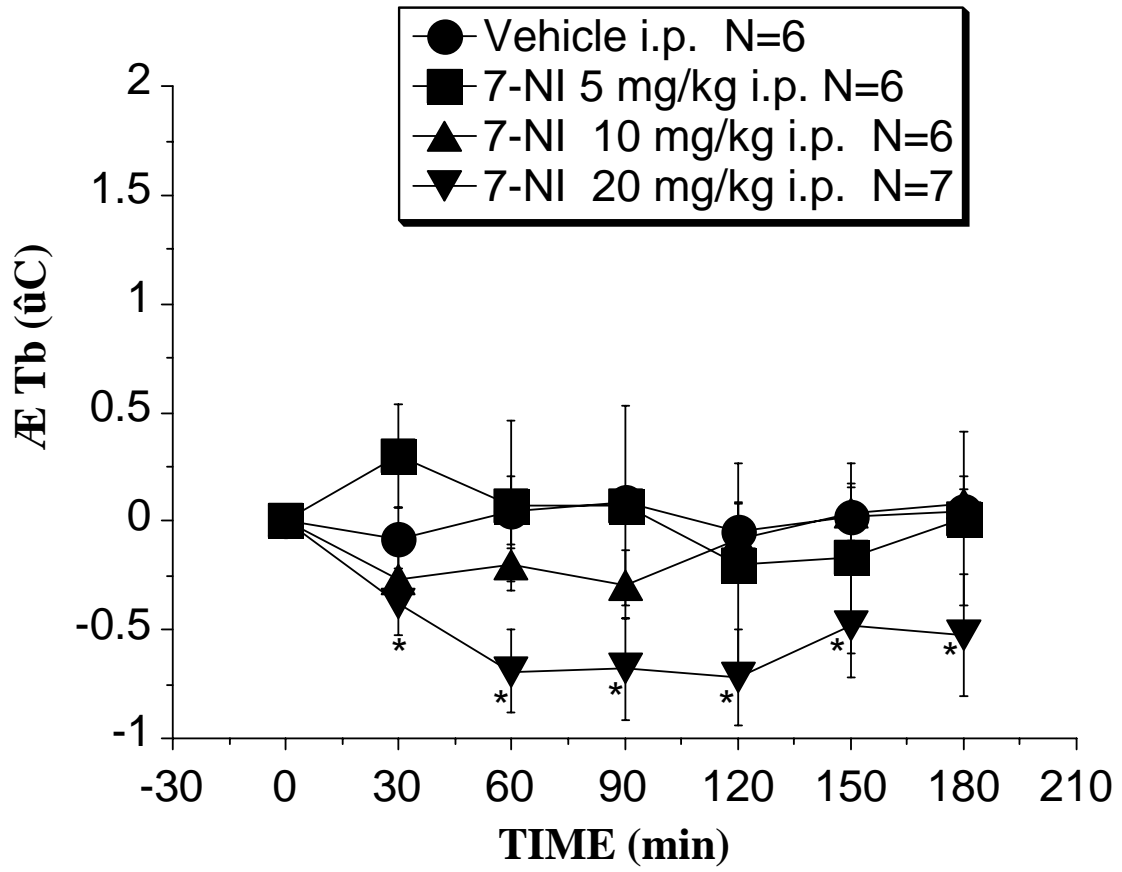


FIGURE 2

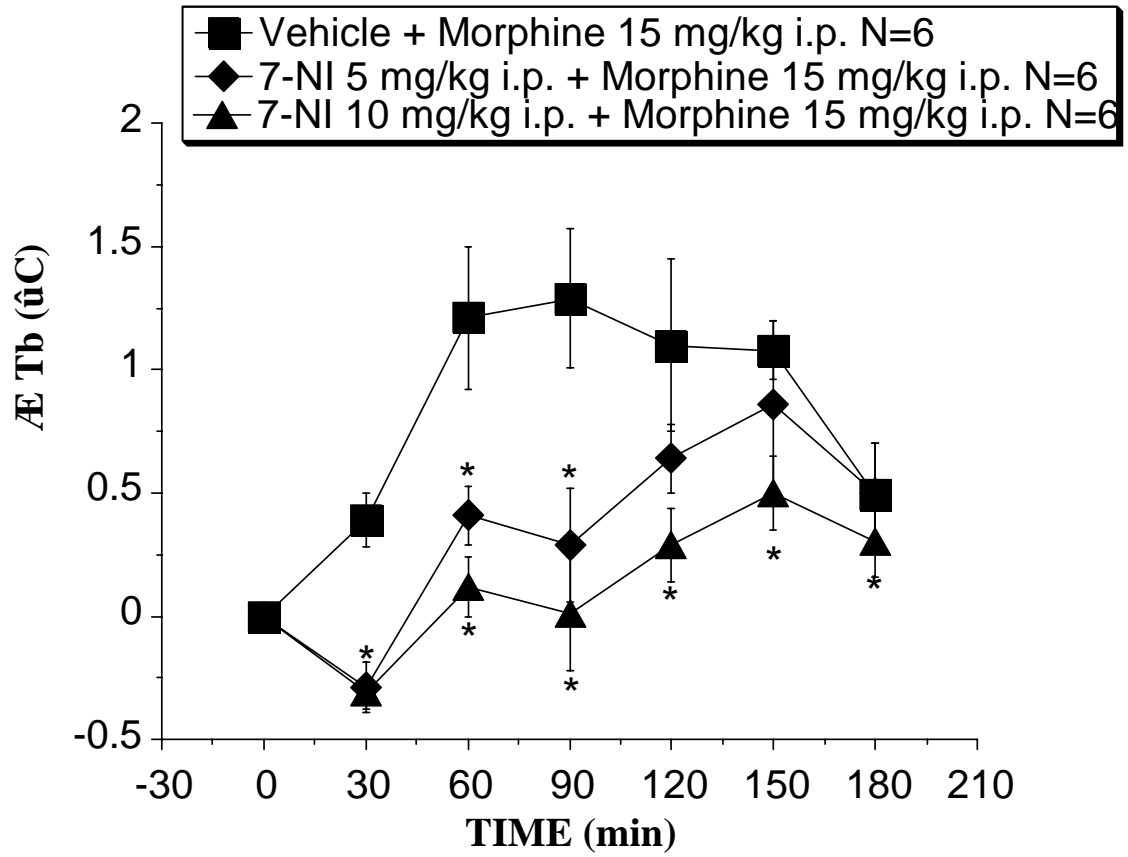


FIGURE 3

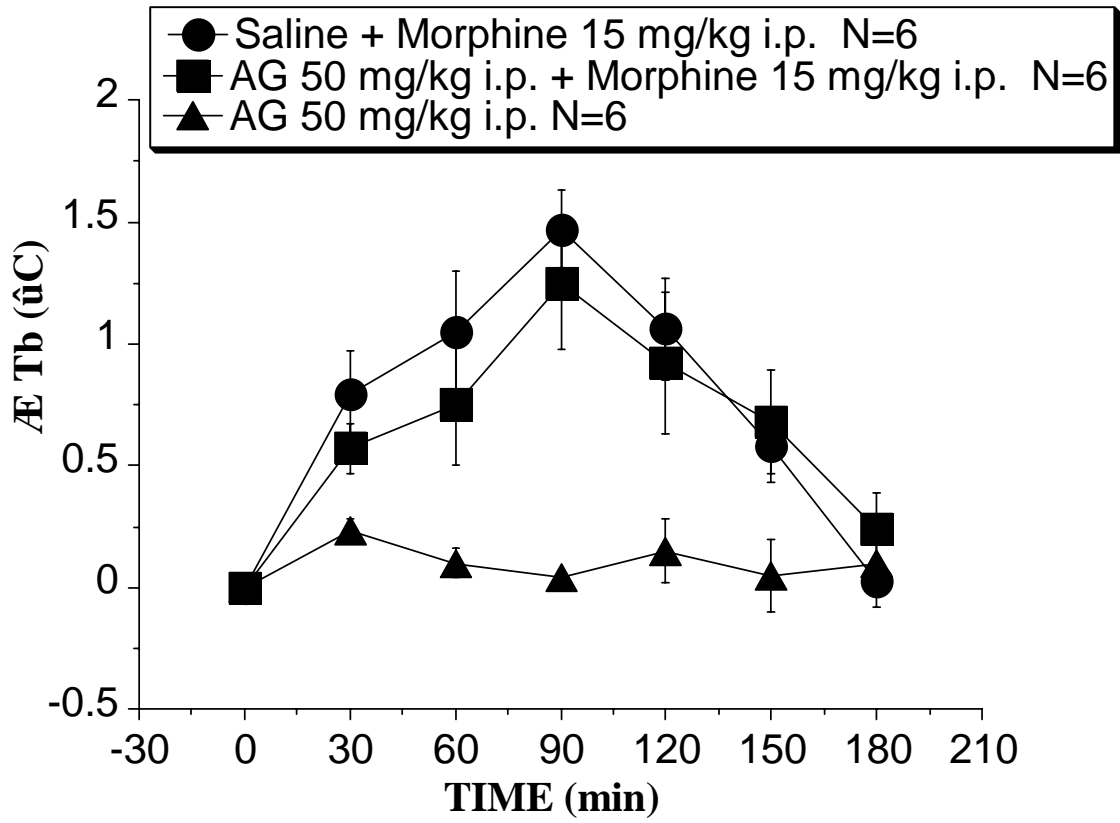


FIGURE 4

