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The impact of advanced opioid drugs and analgesic adjuvants on murine macrophage oxygen burst

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Abstract: Macrophages (Mf) are a versatile group of phagocytic cells responsible for fulfilling a variety of immune functions, most notably for mounting the initial anti-microbial response and for the clearance of cellular debris and apoptotic bodies. The key processes for fulfilling these functions include the production of reactive oxygen intermediates (ROIs) and nitric oxide (NO). Mf also express a variety of receptors, including opioid, serotonin, and norepinephrine receptors, and thus can react to various substances. Our study aimed to examine the effects of oxycodone and buprenorphine on the production ROIs and NO by Mf from intraperitoneally-treated mice, as compared to the previously studied morphine, fentanyl, and methadone, as well as the effects of the analgesic adjuvants gabapentin, amitriptyline, and venlafaxine. ROIs was estimated via luminol and lucigenin dependent chemiluminescence assay, and NO secretion was estimated via a colorimetric method utilizing a modified Griess reaction. We observed an overall decrease in both ROIs and NO production by Mf from adjuvant-treated mice, especially with amitriptyline. Opioids, however, resulted in enhanced ROIs production and mixed NO secretion, with oxycodone and buprenorphine have the least immunomodulatory effects. As ROIs and NO are potent mediators of Mf activity during the innate immune response, our current results express great translational potential. Our results suggest that OPs administration may boost Mf anti-microbial response. On the other hand, during sterile inflammation, enhanced generation of ROIs by Mf influenced by opioids may increase the risk of tissue damage, but co-administration of adjuvants could abolish this adverse effect.

Key words: opioid, amitriptyline, venlafaxine, gabapentin, innate immunity, pain, reactive oxygen intermediates, nitric oxide.

Introduction

Macrophages (Mf) are a versatile group of phagocytic cells responsible for fulfilling a variety of immune functions, most notably for mounting the initial anti-microbial response and for the clearance of cellular debris and apoptotic bodies. Thus, tissue resident Mf play an important role in innate immunity, as they are able either to activate and promote acute inflammation against pathogen or to counteract immune response against self-antigens. Additionally, Mf function as antigen presenting and effector cells, helping to induce the adaptive, humoral, and cellular immune response [1–2]. Due to their ability to release various pro and anti-inflammatory mediators, Mf also contribute to the orchestration and regulation of immune mechanisms, and their cytotoxic activation is observed in delayed-type hypersensitivity (DTH), including the cutaneous contact sensitivity (CS) reaction. The key processes for fulfilling this variety of functions include the production of reactive oxygen intermediates (ROIs) and nitric oxide (NO) [3–5]. It is also notable that Mf express a variety of cell-membrane--associated and intracellular receptors, including opioid, serotonin, and norepinephrine receptors [6–11]. Thus, these cells can react to numerous signaling molecules, including cytokines, hormones, neurotransmitters and bioactive compounds of different medications [12].

As mentioned above, Mf cytotoxic activity is facilitated mostly by the release of reactive oxygen intermediates (ROIs) and nitric oxide (NO). When induced by pathogen infection, ROIs and NO are potent microbicidal agents that favor the activation of acute inflammation. However, during chronic or sterile inflammation, Mf-derived oxygen and nitrogen radicals could exacerbate the oxidative stress responsible for tissue damage and oxidative modifications of self-proteins and lipids. On the other hand, during the adaptive immune response, secreted NO greatly increases Mf cytotoxic activity against intracellular pathogens inducing DTH, and together with ROIs, could regulate immune mechanisms through induction of effector T cell apoptosis and stimulation of regulatory T cell function [13]. The zymosan-stimulated generation of ROIs by Mf can be measured in vitro in the luminol and lucigenin dependent chemiluminescence assay. As luminol may cross cellular membranes, it can be oxidized by both extra- and intracellular pools of ROIs to emit luminescence, while lucigenin is oxidized only by extracellularly secreted ROIs [13, 14]. Further, the concentration of NO secreted to culture medium by Mf stimulated with bacterial LPS can be measured in a colorimetric method based on Griess reaction.

Opioid titration is a common procedure in the management of unrelieved pain. In some instances, increasing the opioid dose leads to a paradoxical intensification of the pain experience. This phenomenon is named opioid hyperalgesia [15]. Additionally, opioid drug treatment of neuropathic pain is significantly less effective, since their analgesic action appears later than exerted severe adverse effects. Accordingly, the use of analgesic adjuvants (AAs) should be considered for the alleviation of uncontrolled pain, especially of a neuropathic nature [16] and for mitigation of opioid-induced hyperalgesia [15]. The adjuvant amplification of opioid action has already been shown in the case of some of antidepressants and anticonvulsants. Analgesic properties of opioids but also AAs, among other things depend on their direct influence on function of immune cells. Targeting of macrophages by analgesic drugs could be the most effective.

Filipczak-Bryniarska *et al.* previously showed enhancement of ROIs production in mice treated with opioids (OPs), such as morphine, fentanyl, and methadone [14]. Yet, little has been demonstrated regarding the immunological effects of two other commonly used OPs, namely oxycodone and buprenorphine. Oxycodone is a commonly used opioid, which acts mainly as a μ-receptor agonist, but is also believed to have some κ-receptor activity [17–19]. Buprenorphine is a semisynthetic, highly lipophilic opioid, about 30 times stronger than morphine, which acts as a μ-receptor agonist, as well as a κ-receptor antagonist [17, 19, 20].

In the palliative care setting, OPs are often administered with AAs. AAs are drugs, which are not expressly indicated to treat pain, but have been found to be useful in certain clinical situations, such as in the treatment of neuropathic pain [19]. Some of the most commonly used AAs include anticonvulsants, such as gabapentin (GABA); tricyclic antidepressants, such as amitriptyline (AMI); and serotonin-norepinephrine reuptake inhibitors, such as venlafaxine (VENLA). While the analgesic efficacy of OPs and AAs is well established, much remains to be elucidated about their effects on the immune system [19, 21].

Our study aimed to examine the effects of oxycodone and buprenorphine on the production ROIs and NO by murine Mf, as compared to the previously studied morphine, fentanyl, and methadone, as well as the effects of GABA, AMI, and VENLA, both independently and with co-administration of the prototypic opioid morphine.

Materials and methods

Animals

For this study, we used inbred male CBA/J mice, ranging from eight to ten weeks old, which were procured from the Animal Facility of the Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland. All animal subjects were given free

access to food and water. All experiments were conducted according to the guidelines of the Animal Care and Use Committee of the Jagiellonian University Medical College and under ethical approval of the 1st Local Ethics Committee (approval no. 102/2009, 123/2013, 215/2015).

Drugs

The following opioid drugs were used: buprenorphine (01AF0512), fentanyl (06BC0815), morphine sulfate (02DR0910), naloxone hydrochloride (02BZ0514) (WZF Polfa SA, Warsaw, Poland), methadone hydrochloride (Molteni Farmaceutici Polska), oxycodone hydrochloride (AB465, Mundipharma Polska Sp. z o.o., Warsaw, Poland). The following analgesic adjuvant drugs were also used: amitriptyline hydrochloride (A8404), gabapentin (G154) and venlafaxine hydrochloride (V7264) (Sigma, St. Louis, MO, USA). All drugs were used as sterile solutions in DPBS for intraperitoneal injections.

Reagents

The following reagents were used: acetone, ethanol, glucose, reagent kit for Griess reaction, trypan blue (POCh SA, Gliwice, Poland), Dulbecco's phosphate-buffered saline (DPBS), fetal calf serum (FCS), Pen-Strep, RPMI1640 (Gibco Life Technologies, Grand Island, NY, USA), dimethyl sulfoxide (DMSO), heparin sodium salt, lucigenin (bis-methylacridinum nitrate), luminol (3-aminophthalic hydrazide), 2-mercaptoethanol (2-ME), mineral oil (heavy fraction), TRIS buffer, zymosan (freshly opsonized with mouse serum) (Sigma, St. Louis, MO, USA) and lipopolysaccharide (LPS) (BIO-Whittaker, Walkersville, MD).

Experimental design

This study was divided into two sets of experiments, the first involving only OPs and the second involving morphine (as the most studied opioid representative) and AAs. Oil-induced peritoneal Mf were harvested from mouse donors, who had been treated intraperitoneally (i.p.) with OPs and/or AAs. In the first group of experiments, mice were treated for seven days with the following drugs: morphine at a dose of 20 mg/kg twice per 24 h, fentanyl 10 mg/kg twice per 24 h, methadone 30 mg/kg once per 24 h, oxycodone 20 mg/kg twice per 24 h, buprenorphine 2 mg/kg once per 24 h, and naloxone 1 mg/kg once per 24 h. In the second group of experiments, mice were treated for eight days with morphine; morphine and naloxone; AMI; AMI and morphine; GABA; GABA and morphine; VENLA; or VENLA and morphine. Dosages were as follows: morphine 20 mg/kg twice per 24 h, naloxone 1 mg/kg once per 24 h,

AMI 10 mg/kg once per 24 h, GABA 5 mg/kg once per 24 h, VENLA 5 mg/kg once per 24 h. In both cases some mice did not receive any medicaments and were used as a control. Mf were induced by the i.p. injection of 1 ml of mineral oil on day 2 for the OPs-only group of experiments and on day 3 for the morphine and AAs group.

Harvesting of oil-induced peritoneal Mf

Mf were harvested from peritoneal cavities of mice 5 days after injection with mineral oil. Each treatment group was represented by Mf from 3 to 6 mice. Mf were washed out of peritoneal cavities with 5 ml of ice-cold DPBS containing heparin (5 U/ml) and, after control of their viability, used for in vitro studies.

Measurements of ROIs production

The production of ROIs by Mf from mice in all treatment groups, as well as from control mice, was analyzed by a luminol-dependent and lucigenin-dependent chemiluminescence assay. Mf from all treatment and control groups were added to 96-well flat bottom dark plates at a concentration of 5×10^5 viable cells/well in 0.2 ml RPMI1640 medium supplemented with 10% FCS. Chemiluminescence probes were then added and Mf were incubated in darkness for 15 min at 37°C. Mouse serum-opsonized zymosan was subsequently added to selected wells, and plates were immediately transferred to a Lucy 1 luminometer (Anthos, Salzburg, Austria). The measurement of luminescence lasted for 75 min. Each measurement was completed twice.

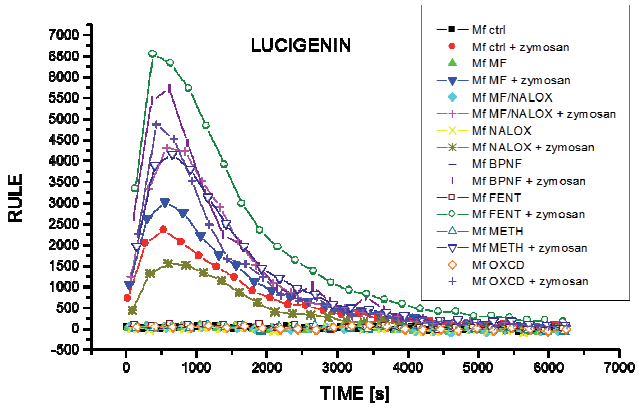
Measurement of nitric oxide concentration

Mf from the various treatment and control groups were placed in wells of 24-well plate at a concentration of 5×10^5/ml in RPMI1640 medium supplemented with 5% FCS and cultured in 5% CO_2 at a temperature of 37°C. Lipopolysaccharide (LPS) was added to some wells to achieve a concentration of 100 ng/ml. Supernatants for NO measurement were collected after 24 h of culture. Concentrations of NO were immediately measured via a colorimetric method utilizing a modified Griess reaction [13].

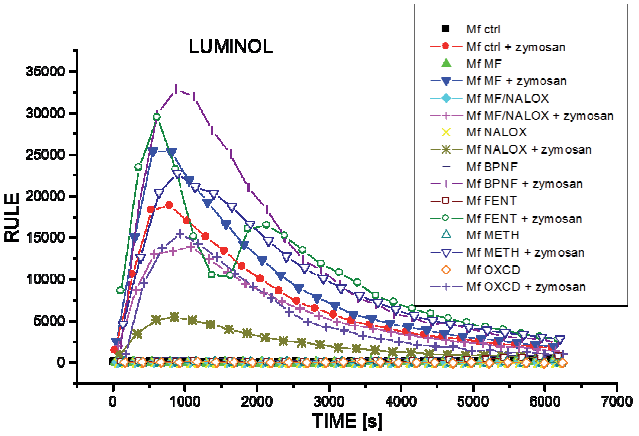
Results

Effect of OPs on zymosan-induced ROIs production

When measuring extracellular ROIs generation using lucigenin, which is too large to enter Mf [22, 23], we observed a significant increase in ROIs production by Mf isolated from mice treated with OPs, in particular fentanyl (peak about 6,500), buprenorphine



(a)



(b)

Fig. 1. The influence of opioid administration on the production of reactive oxygen intermediates (ROIs) by macrophages. Oil-induced peritoneal macrophages from mice treated for seven days with each respective opioid were incubated at 37°C for 15 min with a chemiluminescence probe, either lucigenin (a) or luminol (b), on 96-well flat bottom dark plates at a concentration of 5×10^5 viable cells/well in 0.2 ml RPMI1640 medium supplemented with 10% FCS. Samples were subsequently stimulated with zymosan and the kinetics of ROIs production was measured via chemiluminescence measurement in a luminometer. Results are expressed in relative unites of luminescence emission (RULE) per second.
Mf ctrl: untreated control macrophages; Mf MF: macrophages from mice treated with morphine; Mf MF/NALOX: macrophages from mice treated with morphine and naloxone; Mf BPNF: macrophages from mice treated with buprenorphine; Mf FENT: macrophages from mice treated with fentanyl; Mf METH: macrophages from mice treated with methadone; Mf OXCD: macrophages from mice treated with oxycodone.

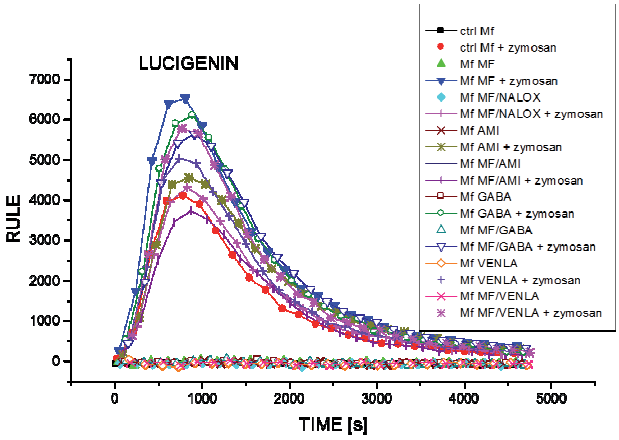
(peak about 5,750), and oxycodone (peak about 4,800), when compared to production by Mf from control mice (peak about 2,400) (Fig. 1a). When measuring extra- and intracellular ROIs generation using luminol, which is able to cross cell membranes [22, 23], we again observed a significant increase in their production by Mf isolated from mice treated with OPs, when compared to production by Mf from control mice (peak about 19,000) (Fig. 1b). Fentanyl and buprenorphine were again the most stimulatory. Interestingly, we observed decreased total ROIs production by Mf from mice who had received oxycodone (peak about 15,500) when compared to control mice. Notably, Mf from mice treated with naloxone solely expressed significantly weakened production of ROIs in comparison to Mf from drug-untreated or morphine-treated mice. Similarly, Mf from mice treated with morphine + naloxone showed decreased ROIs production when compared to Mf from morphine-treated donors.

Effect of AAs on zymosan-induced ROIs production

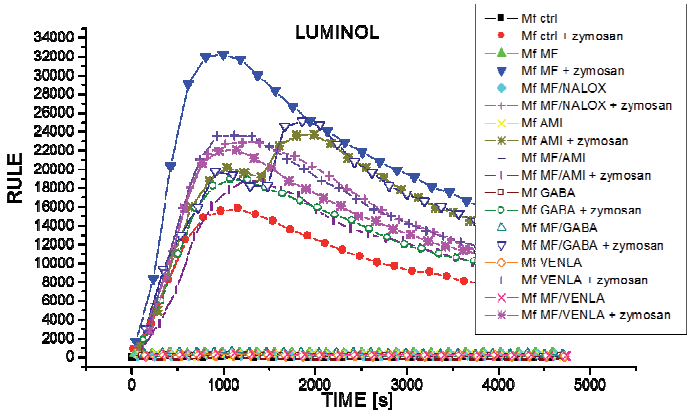
When measuring extracellular ROIs generation using lucigenin, we observed a significant decrease in ROIs production by Mf isolated from mice treated with AAs, particularly from mice treated solely with amitriptyline (peak about 4,500) and venlafaxine (peak about 5,000), as compared to Mf from mice treated with morphine (peak about 6,500) (Fig. 2a). Quite intriguing was our observation that Mf from mice who received morphine + AA showed decreased production of ROIs in comparison with Mf from morphine-treated donors, and in the case of Mf from mice administered with morphine + amitriptyline even when compared with control Mf. When measuring total ROIs generation using luminol, we observed a similar decrease in peak ROIs production from AA-treated mice, as compared to morphine-treated animals (Fig. 2b). Furthermore, co-treatment of Mf donors with morphine + AA again resulted in reduced generation of total pool of ROIs by Mf when compared to effects of treatment with morphine alone. The greatest decrease was also seen in mice treated with morphine + amitriptyline (peak about 18,000), when compared to mice who received only morphine (peak about 32,000). However, total peak ROIs production by these Mf was higher than in control Mf. As above, an analogous inhibitory effect on ROIs generation was shown in the case of Mf from donors administered with morphine + naloxone, when compared to morphine-treated donor Mf.

Effect of OPs on NO secretion

When estimating the concentration of NO in supernatants from cultures of LPS-stimulated Mf from mice treated with OPs, we observed some quite interesting results (Table 1). We demonstrated an increased concentration of NO in samples of



(a)



(b)

Fig. 2. The influence of analgesic adjuvant administration on the production of reactive oxygen intermediates (ROIs) by macrophages. Oil-induced peritoneal macrophages from mice treated for seven days with each analgesic adjuvant, with or without co-administration of morphine, were incubated at 37°C for 15 min with a chemiluminescence probe, either lucigenin (a) or luminol (b), on 96-well flat bottom dark plates at a concentration of 5×10^5 viable cells/well in 0.2 ml RPMI1640 medium supplemented with 10% FCS. Samples were subsequently stimulated with zymosan and the kinetics of ROIs production was measured via chemiluminescence measurement in a luminometer. Results are expressed in relative unites of luminescence emission (RULE) per second.
Mf ctrl: untreated control macrophages; Mf MF: macrophages from mice treated with morphine; Mf MF/NALOX: macrophages from mice treated with morphine and naloxone; Mf AMI: macrophages from mice treated amitriptyline; Mf MF/AMI: macrophages from mice treated with morphine and amitriptyline; Mf GABA: macrophages from mice treated with gabapentin; Mf MF/GABA: macrophages from mice treated with morphine and gabapentin; Mf VENLA: macrophages from mice treated with venlafaxine; Mf MF/VENLA: macrophages from mice treated with morphine and venlafaxine.

Mf isolated from mice treated with morphine and buprenorphine, but we observed a greatly decreased concentration in those of mice treated with fentanyl, methadone, and oxycodone. This pattern was also seen in the concentration of unstimulated samples, with the exception of morphine-treated mice, whose Mf exhibited a decreased NO concentration compared to those of control mice. In addition, Mf from mice treated with morphine + naloxone released less NO than those from donors treated with morphine alone and its supernatant concentration was comparable to this of control Mf.

Table 1. The influence of opioids on nitric oxide secretion by macrophages.

Group	Unstimulated Mf [10^5/mL]	Mf stimulated *in vitro* with LPS [100 ng/mL]
Mf ctrl	5.6 ± 0.3	17.20 ± 0.4
Mf MF	2.1 ± 0.3	26.90 ± 4.2
Mf MF/NALOX	3.6 ± 0.3	17.70 ± 2.3
Mf NALOX	4.5 ± 1.4	15.20 ± 1.8
Mf BPNF	7.5 ± 2.0	20.80 ± 2.1
Mf FENT	1.7 ± 0.1	2.04 ± 0.1
Mf METH	1.8 ± 0.2	4.54 ± 1.2
Mf OXCD	3.8 ± 0.1	9.40 ± 0.4

Oil-induced peritoneal macrophages from mice treated for seven days with each respective opioid were placed in wells of a 24-well plate at a concentration of 5 × 10^5/ml in RPMI1640 medium supplemented with 5% FCS and cultured in 5% CO_2 at a temperature of 37°C. Lipopolysaccharide (LPS) was added to some wells to achieve a concentration of 100 ng/ml. Supernatants for nitric oxide measurement were collected after 24 h of culture. Concentrations of NO were immediately measured via a colorimetric method utilizing a modified Griess reaction. Nitric oxide concentrations are expressed in μM.

Mf ctrl: untreated control macrophages; Mf MF: macrophages from mice treated with morphine; Mf MF/NALOX: macrophages from mice treated with morphine and naloxone; Mf BPNF: macrophages from mice treated with buprenorphine; Mf FENT: macrophages from mice treated with fentanyl; Mf METH: macrophages from mice treated with methadone; Mf OXCD: macrophages from mice treated with oxycodone.

Effects of AAs on NO secretion

When unstimulated with LPS, all AA-treated groups showed a decreased basal secretion of NO compared to the control group (Table 2). When stimulated with LPS, we observed a significantly decreased secretion in groups of Mf from mice treated with amitriptyline, morphine + amitriptyline, gabapentin and morphine + gabapentin. Interestingly, we observed an increased secretion by Mf from mice treated with venlafaxine + morphine, and a secretion comparable to control Mf from venlafaxine-treated animals.

Table 2. The influence of analgesic adjuvants on nitric oxide secretion by macrophages.

Group	Unstimulated Mf [10^5/mL]	Mf stimulated *in vitro* with LPS [100 ng/mL]
Mf ctrl	5.6 ± 0.30	17.2 ± 0.4
Mf MF	2.1 ± 0.30	26.9 ± 4.2
Mf MF/NALOX	3.6 ± 0.30	17.7 ± 2.3
Mf AMI	0.0 ± 0.00	3.0 ± 2.5
Mf MF/AMI	0.0 ± 0.00	4.0 ± 1.0
Mf GABA	0.0 ± 0.00	7.0 ± 2.0
Mf MF/GABA	0.0 ± 0.20	12.0 ± 1.5
Mf VENLA	0.1 ± 0.05	17.1 ± 2.1
Mf MF/VENLA	3.8 ± 0.10	24.0 ± 3.2

Oil-induced peritoneal macrophages from mice treated for seven days with each respective analgesic adjuvant, with or without co-administration of morphine, were placed in wells of a 24-well plate at a concentration of 5×10^5/ml in RPMI1640 medium supplemented with 5% FCS and cultured in 5% CO_2 at a temperature of 37°C. Lipopolysaccharide (LPS) was added to some wells to achieve a concentration of 100 ng/ml. Supernatants for nitric oxide measurement were collected after 24 h of culture. Concentrations of NO were immediately measured via a colorimetric method utilizing a modified Griess reaction. Nitric oxide concentrations are expressed in μM.

Mf ctrl: untreated control macrophages; Mf MF: macrophages from mice treated with morphine; Mf MF/NALOX: macrophages from mice treated with morphine and naloxone; Mf AMI: macrophages from mice treated amitriptyline; Mf MF/AMI: macrophages from mice treated with morphine and amitriptyline; Mf GABA: macrophages from mice treated with gabapentin; Mf MF/GABA: macrophages from mice treated with morphine and gabapentin; Mf VENLA: macrophages from mice treated with venlafaxine; Mf MF/VENLA: macrophages from mice treated with morphine and venlafaxine.

Discussion

Current results showed that week-long treatment of mice with OPs, such as morphine, buprenorphine, fentanyl and methadone, led to the enhancement of ROIs generation by Mf activated with zymosan, a fungal pathogen-associated molecular pattern (PAMP) bearing inducer of Mf oxidative burst, as well as a potent inflammatory response [24]. In the case of oxycodone administration, it resulted in the enhancement of generation of extracellular ROIs, while total ROIs production by Mf was slightly reduced. The increase of ROIs generation by Mf induced by morphine treatment was restrained by co-administration of naloxone with morphine. Importantly, co-administration of AAs, such as amitriptyline, gabapentin and venlafaxine, together with morphine also potently moderated the increase of ROIs production by Mf observed as a result of morphine action. In a similar manner, Mf from mice treated with morphine and buprenorphine secreted more NO in a response to LPS stimulation, and this effect was

again reversed by co-administration of naloxone together with morphine. However, LPS-stimulated Mf from animals treated with fentanyl, methadone, and oxycodone were characterized by inhibited release of NO. Importantly, both co-treatment of mice with morphine together with AAs or with AAs solely, also led to a significant reduction of NO release by Mf.

As ROIs and NO are potent mediators of Mf activity in the innate immune response, our current results express great translational potential. Accordingly, it could be speculated that OPs administration may boost Mf anti-microbial response. On the other hand, during sterile inflammation, enhanced generation of ROIs by Mf influenced by opioid drugs may increase the risk of tissue damage, and co-administration of AAs could likely negate this adverse effect.

However, during sterile inflammation, ROIs secreted by Mf were also shown to control and finally diminish the active immune response by targeting effector T lymphocytes, which leads to the inhibition of their differentiation along with induction of their apoptosis [25]. Further, this ROIs-mediated mechanism of immune response restriction additionally promotes the survival of memory T cells [25]. Importantly, due to the very short biological half-life of ROIs, their activity is limited to cells in close proximity to Mf [26], especially those forming the immune synapse [26, 27]. ROIs-mediated effects may either significantly affect intracellular signalization pathways [28], or, along with cytokine-induced effects, might activate the effector response at the immune synapse [29]. Thus, our current observations suggest that OPs-induced effects on ROIs generation by Mf may facilitate both the activation of cells at the immune synapse and the naturally occurring process of limitation of the immune response to the required time and intensity. Interestingly, mice with genetically restricted ROIs deficiency were shown to express enhanced risk of developing an autoimmune response [30]. Accordingly, the protective effects of ROIs generated by Mf in the course of rheumatoid arthritis was demonstrated to be associated with the activation of regulatory T lymphocytes [31, 32]. Therefore, OPs administration could augment the beneficial effects of Mf-derived ROIs in prevention of autoimmune responses through the activation of regulatory cells.

Mf-derived NO was also identified as a potent regulator of immune response, involved in the suppression of T cell-dependent immunity [33–36], including the CS reaction [37]. Thus, while treatment with morphine or buprenorphine may additionally support the restriction of excessive inflammatory and allergic or hypersensitive response by enhancing NO production by Mf, administration of other OPs, especially together with AAs, may impair the regulation of the T cell-mediated immune response.

Of clinical interest, the production of ROIs and NO are essential processes performed by Mf during the innate immune response. Their production assists Mf in fulfilling a variety of functions, ranging from the killing of microbes to antigen

processing [1–4]. While being invaluable for the proper functioning of the immune system, their overproduction has also been implicated in a number of harmful processes, including the cachexia-anorexia syndrome (CAS) [19, 38–40]. CAS is characterized clinically by the involuntary loss of fat and muscle tissue, as well as loss of appetite [19]. Although the pathophysiology of this syndrome is not fully understood, it is thought to result from generalized hormonal and immune dysfunction. Several specific immune-related substances are implicated in its pathogenesis, including ROIs. Other animal studies have implicated ROIs and NO in muscle damage and wasting [41–43]. Furthermore, some clinical studies in humans have found antioxidant treatment to be effective in limiting muscle wasting among cancer patients [44].

The results of our study suggest a strong enhancement of the production of ROIs by Mf from mice treated with OPs, which is in line with previous research [14]. It is important to note that OPs are necessary for pain control in up to two-thirds of patients suffering from advanced neoplastic disease [19]. These advanced cancer patients are also the most at risk for the development of CAS. However, our study also found that the addition of an AA to OPs treatment greatly limited the enhancement of ROIs production. This was particularly pronounced in the case of amitriptyline. We also found that AAs led to a lower basal secretion of NO, and amitriptyline led to decreased NO secretion even when Mf were stimulated with LPS. Based upon these observations, AAs may be beneficial additions to the treatment regimen of patients receiving OPs, especially those suffering from or at risk of cachexia. Further studies are warranted to investigate their therapeutic potential, with amitriptyline being a particularly suitable candidate for further investigation.

Tricyclic antidepressants, herein represented by amitriptyline, act as serotonin re-uptake inhibitors, similarly to venlafaxine, which additionally blocks norepinephrine re-uptake. As macrophages express receptors for serotonin and norepinephrine, they can sense the clinically effective increase in the concentration of neurotransmitters [45]. Thus, it could be suspected that neurotransmitter-induced effects are also involved in immune-mediated analgesia by antidepressant AAs. Further, the inhibition of macrophage release of proinflammatory cytokines, such as IL-6 and TNFα, was recently demonstrated as a result of venlafaxine and imipramine administration [46]. As these cytokines strongly contribute to the immune inducing mechanisms of severe neuropathic pain, their inhibition likely expresses an analgesic effect that could also be responsible for the antidepressant action of AAs.

The anticonvulsant gabapentin ameliorates pathological signaling through the ion channels. It binds to the G protein of voltage-dependent calcium channel, which leads to pain relieving cell hyperpolarization. This effect of gabapentin was recently shown to reverse proinflammatory activation of macrophages observed in chemotherapy-induced peripheral neuropathy [47]. Similarly, our current results

show that co-treatment with morphine and gabapentin reduced the oxidative burst of macrophages strengthened by treatment with morphine alone.

In conclusion, it seems to be important to point out that, in comparison to morphine, buprenorphine and oxycodone express weaker immunomodulatory properties, avoiding redundant suppression of physiological immune defense. Increasing opioid doses often is insufficient and can produce hyperalgesia in the treatment of severe pain. Thus, the addition of AAs to opioid therapy, facilitating immune mechanisms responsible for pain relief, possesses great clinical importance.

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Conflict of interest

None declared.

Disclosures

Michael Kozlowski planned and performed the experiments and wrote the manuscript; Katarzyna Nazimek performed the experiments and revised the manuscript; Magdalena Wąsik supported performance of experiments; Iwona Filipczak-Bryniarska consulted and assisted in planning and performance of experiments, revised the manuscript and supervised Michael Kozlowski; Krzysztof Bryniarski consulted and assisted in planning and performance of experiments, revised the manuscript and supervised Michael Kozlowski and Magdalena Wąsik.

Abbreviations

Mf — macrophages
ROIs — reactive oxygen intermediates
NO — nitric oxide
OPs — opioids
AA — analgesic adjuvant
GABA — gabapentin
AMI — amitriptyline
VENLA — venlafaxine
MF — morphine
NALOX — naloxone

BPNF — buprenorphine
FENT — fentanyl
METH — methadone
OXCD — oxycodone
ctrl — control
LPS — lipopolysaccharide
CAS — cachexia anorexia syndrome

References

1. *Bryniarski K., Szczepanik M., Maresz K., Ptak M., Ptak W.*: Subpopulations of mouse testicular macrophages and their immunoregulatory function. Am J Reprod Immunol. 2004; 52: 27–35.
2. *Hughes C.E., Benson R.A., Bedaj M., Maffia P.*: Antigen-Presenting Cells and Antigen Presentation in Tertiary Lymphoid Organs. Front Immunol. 2016; 7: 481.
3. *Dupré-Crochet S., Erard M., Nüβe O.*: ROS production in phagocytes: why, when, and where? J Leukoc Biol. 2013; 94: 657–670.
4. *Nazimek K., Bryniarski K.*: The biological activity of macrophages in health and disease. Postepy Hig Med Dosw. 2012; 66: 507–520.
5. *Esquivel-Solís H., Vallecillo A.J., Benítez-Guzmán A., Adams L.G., López-Vidal Y., Gutiérrez--Pabello J.A.*: Nitric oxide not apoptosis mediates differential killing of Mycobacterium bovis in bovine macrophages. PLoS ONE. 2013; 8: e63464.
6. *Delgado-Vélez M., Lugo-Chinchilla A., Lizardo L., et al.*: Chronic exposure of human macrophages in vitro to morphine and methadone induces a putative tolerant/dependent state. J Neuroimmunol. 2008; 196: 94–100.
7. *Vallejo R., de Leon-Casasola O., Benyamin R.*: Opioid therapy and immunosuppression. Am J Ther. 2004; 11: 354–365.
8. *Franchi S., Moretti S., Castelli M., et al.*.: Mu opioid receptor activation modulates Toll like receptor 4 in murine macrophages. Brain Behav Immun. 2012; 26: 480–488.
9. *Tomassini N., Renaud F., Roy S., Loh H.H.*: Morphine inhibits Fc-mediated phagocytosis through mu and delta opioid receptors. J Neuroimmunol. 2004; 147: 131–133.
10. *Mikulski Z., Zaslona Z., Cakarova L., et al.*: Serotonin activates murine alveolar macrophages through 5-HT2C receptors. Am J Physiol Lung Cell Mol Physiol. 2010; 299: L272–L280.
11. *Huang J.L., Zhang Y.L., Wang C.C., et al.*: Enhanced phosphorylation of MAPKs by NE promotes TNF-α production by macrophage through α adrenergic receptor. Inflammation. 2012; 35: 527–534.
12. *Nazimek K., Filipczak-Bryniarska I., Bryniarski K.*: The role of medicaments, exosomes and miRNA molecules in modulation of macrophage immune activity. Postepy Hig Med Dosw. 2015; 69: 1114–1129.
13. *Nazimek K., Nowak B., Marcinkiewicz J., Ptak M., Ptak W., Bryniarski K.*: Enhanced generation of reactive oxygen intermediates by suppressor T cell-derived exosome-treated macrophages. Folia Med Cracov. 2014; 54: 37–52.
14. *Filipczak-Bryniarska I., Nowak B., Sikora E., et al.*: The influence of opioids on the humoral and cell--mediated immune responses in mice. The role of macrophages. Pharmacol Rep. 2012; 64: 1200–1215.
15. *Stoicea N., Russel D., Weidner G., et al.*: Opioid-induced hyperalgesia in chronic pain patients and the mitigating effects of gabapentin. Front Pharmacol. 2015; 6: 104.
16. *Ohsawa M., Otake S., Murakami T., Yamamoto S., Makino T., Ono H.*: Gabapentin prevents oxaliplatin-induced mechanical hyperalgesia in mice. J Pharmacol Sci. 2014; 125: 292–299.

17. *Volpe D.A., McMahon Tobin G.A., Mellon R.D., et al.*: Uniform assessment and ranking of opioid μ receptor binding constants for selected opioid drugs. Regul Toxicol Pharmacol. 2011; 59: 385–390.
18. *Nozaki C., Saitoh A., Tamura N., Kamei J.*: Antinociceptive effect of oxycodone in diabetic mice. Eur J Pharmacol. 2005; 524: 75–79.
19. *Bruera E., Higginson I., von Guten C., Morita T.*: Textbook of Palliative Medicine and Supportive Care. Second Edition. CRC Press, Boca Raton, Florida, 2016.
20. *Villiger J.W., Taylor K.M.*: Buprenorphine: characteristics of binding sites in the rat central nervous system. Life Sci. 1981; 29: 2699–2708.
21. Opioids in palliative care: safe and effective prescribing of strong opioids for pain in palliative care of adults. Clinical Guidelines. National Institute for Health and Care Excellence, London, 2012.
22. *Caldefie-Chézet F., Walrand S., Moinard C., Tridon A., Chassagne J., Vasson M.P.*: Is the neutrophil reactive oxygen species production measured by luminol and lucigenin chemiluminescence intra or extracellular? Comparison with DCFH-DA flow cytometry and cytochrome c reduction. Clin Chim Acta. 2002; 319: 9–17.
23. *Kopprasch S., Pietzsch J., Graessler J.*: Validation of different chemilumigenic substrates for detecting extracellular generation of reactive oxygen species by phagocytes and endothelial cells. Luminescence. 2003; 18: 268–273.
24. *Wang H., Wang L., Li N.L., et al.*: Subanesthetic isoflurane reduces zymosan-induced inflammation in murine Kupffer cells by inhibiting ROS-activated p38 MAPK/NF-κB signaling. Oxid Med Cell Longev. 2014; 2014: 851692.
25. *Tripathi P., Hilderman D.*: Sensitization of T cells to apoptosis — a role for ROS? Apoptosis. 2004; 9: 515–523.
26. *Kusmartsev S., Nefedova Y., Yoder D., Gabrilovich D.I.*: Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol. 2004; 172: 989–999.
27. *Hultqvist M., Olsson L.M., Gelderman K.A., Holmdahl R.*: The protective role of ROS in autoimmune disease. Trends Immunol. 2009; 30: 201–208.
28. *Forman H.J., Torres M.*: Signaling by the respiratory burst in macrophages. IUBMB Life. 2001; 51: 365–371.
29. *Tse H.M., Milton M.J., Schreiner S., Profozich J.L., Trucco M., Piganelli J.D.*: Disruption of innate-mediated proinflammatory cytokine and reactive oxygen species third signal leads to antigen-specific hyporesponsiveness. J Immunol. 2007; 178: 908–917.
30. *Hultqvist M., Olofsson P., Holmberg J., Backstrom B.T., Tordsson J., Holmdahl R.*: Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to mutation in the Ncf1 gene. Proc Natl Acad Sci USA. 2004; 101: 12646–12651.
31. *Gelderman K.A., Hultqvist M., Pizzolla A., et al.*: Macrophages suppress T cell responses and arthritis development in mice by producing reactive oxygen species. J Clin Invest. 2007; 117: 3020–3028.
32. *Kraaij M.D., Savage N.D., van der Kooij S.W., et al.*: Induction of regulatory T cells by macrophages is dependent on production of reactive oxygen species. Proc Natl Acad Sci USA. 2010; 107: 17686–17691.
33. *Albina J.E., Abate J.A., Henry W.L. Jr.*: Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-g in the induction of the nitric oxide-synthesizing pathway. J Immunol. 1991; 147: 144–148.
34. *Bingisser R.M., Tilbrook P.A., Holt P.G., Kees U.R.*: Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. J Immunol. 1998; 160: 5729–5734.
35. *Silberman D., Bucknum A., Bartlett T., et al.*: CD28 ligation increases macrophage suppression of T cell proliferation. Cell Mol Immunol. 2012; 9: 341–349.

36. *Silberman D., Bucknum A., Kozlowski M., Matlack R., Riggs J.*: Cytokine treatment of macrophage suppression of T cell activation. Immunobiology. 2010; 215: 70–80.
37. *Ross R., Reske-Kunz A.B.*: The role of NO in contact sensitivity. Int Immunopharmacol. 2001; 1: 1469–1478.
38. *Tisdale M.J.*: Mechanisms of cancer cachexia. Physiol Rev. 2009; 89: 381–410.
39. *Tisdale M.J.*: Cancer cachexia. Curr Opin Gastroenterol. 2010; 26: 146–151.
40. *Grabiec K., Burchert M., Milewska M., Błaszczyk M., Grzelkowska-Kowalczyk K.*: Systemic and local mechanisms leading to cachexia in cancer. Postepy Hig Med Dosw. 2013; 67: 1397–1409.
41. *Guarnier F.A., Cecchini A.L., Suzukawa A.A., et al.*: Time course of skeletal muscle loss and oxidative stress in rats with Walker 256 solid tumor. Muscle Nerve. 2010; 42: 950–958.
42. *Leitner L.M., Wilson R.J., Yan Z., Gödecke A.*: Reactive Oxygen Species/Nitric Oxide Mediated Inter-Organ Communication in Skeletal Muscle Wasting Diseases. Antioxid Redox Signal. 2017; 10.1089/ars.2016.6942.
43. *Zimiani K., Guarnier F.A., Miranda H.C., Watanabe M.A., Cecchini R.*: Nitric oxide mediated oxidative stress injury in rat skeletal muscle subjected to ischemia/reperfusion as evaluated by chemiluminescence. Nitric Oxide. 2005; 13: 196–203.
44. *Mantovani G., Madeddu C., Macciò A., et al.*: Cancer-related anorexia/cachexia syndrome and oxidative stress: an innovative approach beyond current treatment. Cancer Epidemiol Biomarkers Prev. 2004; 13: 1651–1659.
45. *Nazimek K., Strobel S., Bryniarski P., Kozlowski M., Filipczak-Bryniarska I., Bryniarski K.*: The role of macrophages in anti-inflammatory activity of antidepressant drugs. Immunobiology. 2016; doi: 10.1016/j.imbio.2016.07.001.
46. *Nazimek K., Kozlowski M., Bryniarski P., et al.*: Repeatedly administered antidepressant drugs modulate humoral and cellular immune response in mice through action on macrophages. Exp Biol Med (Maywood). 2016; 241: 1540–1550.
47. *Capasso K.E., Manners M.T., Quershi R.A., et al.*: Effect of histone deacetylase inhibitor JNJ-26481585 in pain. J Mol Neurosci. 2015; 55: 570–578.