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Review Article

Oxidative Stress and Opioids

Abstract

In recent years, research has shown the involvement of free radicals in the development of the pain that accompanies many pathological conditions. In the treatment of acute and chronic pain, the most effective therapies are natural and synthetic opioid alkaloids. Their metabolism in itself may contribute to the formation of free radicals and thus affect body system load and the perception of pain. Long-term treatment with opioids is a tool of choice for the treatment of medium and severe pain. Opioids stimulate the effect of endogenous opioids, endorphins, by binding to multiple subtypes of opioid receptors (μ,κ,σ) in spinal, supraspinal and peripheral tissues. Morphine is a typical, natural opioid analgesic utilised in practice in the treatment of severe chronic pain. In addition, similar effects can be expected from semi-synthetic opioids such as oxycodone and hydromorphone. However, during treatment with opioids some adverse effects can appear regardless of whether treatment is short-term or long-term. One potentially serious side effect is the induction of oxidative stress. The purpose of this present work is to determine the main sources of reactive oxygen and nitrogen in the development of inflammatory and neuropathic pain, and the manner in which metabolism of morphine contributes to oxidative stress alone.

Abbreviations

AA: Arachidonic Acid, BH4: Tetrahydrobiopterin, CaM: Calmodulin, CGRP: Calcitonin Gene-Related Peptide, CNS: Central Nervous System, COX: Cyclooxygenase, DUOX: Dual Amino Oxidase, GLUT: Glucose Transporter, HETE: Hydroxyeicosatetraenoic Acid, HPETE: Hydroxyeicosatetraenoic Acid, IL: Interleukin, LC-MS: Liquid Chromatography – Mass Spectrometry, LOX: Lipoxygenase, LT(X_n): Leucotriene, MAPK: Mitogen Activated Proteinkinase, MAPEG: Membrane-Associated Proteins In Eicosanoid And Glutathione Metabolism, NOS: Nitric Oxide Synthase, NOX: NADPH Oxidase Enzymes, NMDA: N-Methyl-D-Aspartate, PG(X_n): Prostaglandin, PLA: Phospholipase, ROS: Reactive Oxygen Species, TNF-A: Tumor Necrosis Factor A, UCP: Uncoupling Protein, UGT: Glucuronosyl Transferase, XDH: Xanthine Dehydrogenase, XO: Xanthine Oxidase, XOR: Xanthine Oxidoreductase

Introduction

Chronic pain is currently one of the major problems and, as such, has lost its defensive physiological importance. Its intensity, duration, and nature do not match the extent of tissue damage. It distorts the psyche of the individual, behaviour, quality of life, undermines the family, social life and, ultimately, presents a socio-economic burden to society. This problem has been so severe, extensive and complex that it required the creation of a new department, known as algesiology. Chronic pain requires a multidisciplinary approach. Examples include non-pharmacological methods - physical therapy, physical therapy and rehabilitation, acupuncture, psychotherapy, psychosocial interventions and relaxation techniques. Another interesting group consists of invasive interventional techniques and neuromodulation processes among others. Despite this, pharmacotherapy has a prominent place in the treatment of chronic pain, which is currently focussed on opioids in the treatment of cancer and severe nonmalignant pain. This treatment brings with it the risk of a variety of side effects, reflected in both the psychological and physical condition of the patient, and at the molecular level. The consequences of these biochemical changes further negatively affect the clinical outcome of the patient. They may become predisposed to torpid progression of previously dormant disease and the emergence of new diseases.

Previous studies provide evidence mainly on the negative effects of opioids on endogenous antioxidant systems. The aim of this summary paper is to highlight the mechanisms by which opioids interfere with the redox mechanisms, occurring in long-term treatment with opioids in patients with chronic pain. The seriousness of the interference should be assessed for the choice of individual adjuvant therapy in patients suffering from chronic pain.

Oxidative stress and pain

Reactive oxygen species (ROS) play an important role in the pathogenesis of disease states [1,2]. Disease-state is characterized by altered metabolic conditions of cells, increased production of ROS, which themselves act as mediators, and other

mediators of inflammation mediating pain expression. ROS are significantly involved in the formation and maintenance of neuropathic pain, which was confirmed by the alleviation of neuropathic pain following administration of non-toxic doses of free oxygen radicals' scavengers [3]. Free radicals are atoms or molecules containing one or more unpaired electron. However, several reactive intermediates are formed from oxygen, not all of them radicals. Therefore, radical and non-radical derivatives of oxygen, including the excited state, and other oxidizing agents of which are easily formed radicals are collectively referred to as the reactive oxygen species (ROS). Due to the many physiological functions in organisms, ROS are normally produced continuously in controllable and uncontrollable processes. Organelles, enzymes and molecules subject to auto-oxidation are all endogenous sources of ROS. The most productive regulated sources include the activities of nitric oxide synthase (NOS), enzymes of NADPH-enzyme family (NOX), lipoxygenase and cyclooxygenase (LOX and COX) as well as the non-adjustable processes of the respiratory chain in mitochondria: xanthine oxidase (XO), autooxidation of dopamine, and photosensitization [4]. Among these are a few fundamental sources directly associated with disease states.

Interconnection between ROS, ATP production in mitochondria and pain: Firstly, we can mention the mitochondrial respiratory chain. Following oxidation of the substrates in the Krebs cycle come electron carriers NADH and $FADH_2$ oxidised at complexes I and II. Reduction equivalents of the released electrons are then transferred through the respiratory chain complex III, and IV by the prosthetic groups arranged according to increasing redox potential to the terminal electron acceptor, which is oxygen. Because of the structure of an oxygen atom, 4 electrons should be simultaneously transferred to the oxygen molecule to allow its reduction to H_2O. The introduction of one electron to an oxygen atom causes its univalent reduction and formation of reactive intermediates. The biggest producers of ROS are complex I and III. Maintaining efficient electron transport to O_2 is a potential mechanism for control of the production of ROS. Regulation of this situation is particularly important in sites of inflammation and triggered immune response. There is, in fact, a significant shift in metabolic activity resulting from a profound recruitment of inflammatory cells (neutrophils and monocytes) and high proliferation rates among lymphocyte populations. The resultant shifts in energy supply and demand (e.g., glucose, oxygen, ATP) can result in metabolic acidosis and diminished delivery and/or availability of oxygen. This can lead to hypoxia extensive enough to trigger transcriptional and translation changes in tissue phenotype [5]. One possible regulatory mechanism is the effect of hexokinases I and II of the ADP:ATP complex (voltage-dependent anion channel). Part of ATP is exported immediately for glucose phosphorylation from mitochondria through the effect of hexokinases. This then enters glycolysis on the mitochondrial surface to form ADP in reverse. Mitochondrial creatine kinase works similarly to the continuous formation of ADP so that the respiratory chain complexes work constantly dragging electrons to complex IV, keeping ATP synthase active and membrane potential low. Inducibility of proton leakage is the major mechanism

of maintaining membrane potential and thus controlling the release of ROS. There is therefore a slight loss of energy (as heat) from the formation of ATP, which can prevent excessive supply of mitochondria by electron/reducing equivalents for the respiratory chain complexes and to minimize the likelihood of interaction of electrons with O_2 and ROS formation. Voltage-dependent anion channels and uncoupling protein UCP1 - 5 (family of mitochondrial anion carriers) permit the escape of protons. However, the ability to catalyse the release of protons which reduce production of ROS was detected only in UCP2 and UCP3. Activation or inhibition of UCPs is controlled by allosteric regulators, which include fatty acids, nucleotides and retinoic acid. Some derivatives of ROS such as O_2^{--} and 4-hydroxynonenal are able to activate UCP2 mediated release of protons. So the overproduction of ROS itself in mitochondria leads to a feedback regulation [6].

In response to oxidative stress and inflammatory conditions, however, the formation and use of energy is important, particularly for phagocytic cells of the immune system that control the resolution of transient inflammatory pain [7]. Cells of myeloid lineages derive their energy almost exclusively from glycolysis, whereas lymphocytes predominantly use oxidative phosphorylation [8,9]. As opposed to lymphocytes that proliferate within tissues, myeloid cells, such as polymorphonuclear leukocytes (neutrophils), macrophages, and dendritic cells, are recruited to sites of inflammation during immune responses. In transit, these cells expend tremendous amounts of energy. Cell migration requires large amounts of actin turnover, which is particularly demanding on ATP [10]. Energy demands of phagocyting cells increase at sites of inflammation. Predominantly using glycolysis for obtaining energy allows them to function at low oxygen concentration and even anoxic conditions associated with deep inflammation. To cover increased energy demands, a glycerol-3-phosphate shuttle is used [9]. Lymphocytes (B and T) use glucose, lipids, and amino acids as energy sources in oxidative phosphorylation. During proliferation, lymphocytes become more glucose dependent, resulting in a nearly 20-fold increase in glucose uptake. This is accomplished by high expression of GLUT-1 [11] tightly controlled by hypoxia-inducible factor, and lactate production can increase 40-fold [12]. In naive T-cells the nutrient uptake is controlled through cytokines (IL-7 and IL-4) [13].

Produced ATP plays an important role in extracellular signalling reactions in inflammation and immune responses. Extracellular ATP (or ADP) can directly bind to cell surface purinergic receptors (P2-type) or can be metabolized to adenosine at the cell surface, where it is made available to bind and activate adenosine receptor(s) (P1-type) [5].

The functions of the P2X family of receptors have previously been associated with pain [14]. Ulmann et al. [15] demonstrate the importance of the ATP-gated calcium channel P2X4 that opens in response to the binding of extra-cellular ATP, and its expression on macrophages, for nociception via upregulation of prostaglandin E_2 (PGE_2).

Involvement of lipoxygenase and cyclooxygenase: Enzymes catalyse the first two reactions of the prostaglandin pathway, leading to the formation intermediate metabolites, prostaglandins G_2 and H_2 (PGG_2 and PGH_2), which are subsequently metabolized by tissue-specific isomerases into prostanoids [16]. The cyclooxygenases (COX-1 and COX-2) and 12, 15, or 5-lipoxygenase (5-LOX) derive prostanoids from arachidonic acid (AA), such as prostaglandin E2 (PGE2) and prostaglandin F2α and 12- and 15-(S)-hydroperoxyeicosatetraenoic acids (HPETE), 5- and 15-(S)-hydroxyeicosatetroenoic acids (HETE), and leukotriene B4. These are potent activators of nociceptors and participate in nociception, or the ability to feel pain [17].

The data available would tend to suggest a role for COX-1 in acute nociception, with inducible COX-2 mechanisms becoming prevalent in more pathological conditions [17]. The synthesis thereof is induced by cytokines and oxidative stress. COX-1 is inducible under inflammatory conditions in the kidney [18], and COX-2 is constitutively expressed in CNS, kidney and blood vessels [19]. As the major inducible isoform, COX-2 is strongly upregulated during inflammation by the products of tissue damage and inflammation such as pro-inflammatory cytokines (IL-1beta, IL-15, TNF-α), and lipopolysaccharides. Indeed, IL-1beta is thought to be one of the major inducers of COX-2 in the CNS under inflammatory conditions [16]. Induction of COX-2 via the MAPK pathway has also been suggested, together with the involvement of peroxynitrite [17]. Interestingly, NO at higher concentrations can suppress induction of COX-2 [20] as can glucocorticoids in the spinal cord [21].

The mechanism of nociception induction is attributed to the mediation of diverse activities through PGE_2 binding to P (1-4) receptors coupled to different signal transduction pathways. While the P1 receptor is coupled to intracellular Ca^{2+} mobilisation, P2 and P4 are coupled to the stimulation of adenylate cyclase. The P2 receptor is also implicated in pain. Harvey et al. [22] hypothesise that activation of the P2 receptor inhibits, by phosphorylation, the glycine receptor (GlyR3), which thus mediates spinal hyperexcitability. The role of P3 and P4 receptors in pain remains to be more clearly explained, as EP3 receptor knockout mice do show reduction in pain behaviours [23]. Conversely, activation of the P4 receptor appears to have an inhibitory function; P4 receptor agonists mediate an inhibition in hyperalgesic scores in an inflammatory pain model [24]. PGE_2 is also able to increase the sensitivity of certain sodium channels, notably those resistant to the effects of tetrodotoxin (TTX). In addition, PGE_2 can modulate the activity of other ion channels, and e.g. inhibits a Ca^{2+}-dependent K+ current, which mediates slow after-hyperpolarisation [17].

In contrast to COX, LOX isoforms also oxidise other lipids, including membrane phospholipids [25]. Oxidized lipids lose their stereospecificity. The oxidation products can include isoprostanes, cholesteryl hydroperoxides, hydroperoxides, epoxides, hydroxycholesterol derivatives, and reactive aldehydes, which enhance the conditions of oxidative stress.

Moreover, arachidonic acid can undergo non-enzymatic (free-radical mediated) oxidation to generate prostaglandin-like products [26]. Oxidatively damaged lipoproteins that can cross-react with antibodies were generated via PGE2 and PGF2 [27]. This finding adds to the speculation that oxidatively-modified lipoproteins can mimic the effects of prostaglandin. A study by Ray et al. [28] demonstrated that lipoproteins modified by non-enzymatic oxidation are similar to prostaglandins in their ability to modulate body temperature, induce nociception and alter the expression of inflammatory and nociceptive genes. LC-MS/MS studies revealed that even non-enzymatic oxidation of LDLs generated both COX and LOX derived oxidation products of polyunsaturated lipids (PGE_2, PGD_2 and 11β-PGE_2 and 12, 15 and 5-HETEs). The likelihood of these molecules playing a role in nociception is not currently known in detail.

Stimulation of NOX enzymes: NOX enzymes are a family of NADPH oxidases (known as NADPH oxidases of phagocytic and not phagocytic cells) that transfer electrons across biological membranes. An example of an electron acceptor would be O_2 producing $O_2^{\cdot-}$. Therefore, their function is to produce ROS and, with respect to the focus of this work, this is also their main role in mediating pain perception.

The massive production of antimicrobial and tumoricidal ROS in an inflammatory environment is called an "oxidative burst" and plays an important role as the first line of defence against environmental pathogens. The combined activities of NADPH oxidase (NOX2 enzymes) and myeloperoxidase (MPO) in phagocytes are an important part of the innate immune response. The activation of phagocytic NADPH oxidase can be induced by microbial products, lipoproteins, or by the cytokines. NAD(P)H oxidase of nonphagocytic cells (NOX1,3-5) is is similar but not identical to phagocytic NADPH oxidase; this can be found in endothelial cells, vascular smooth muscle cells, and cardiac monocytes among others. The rate of $O_2^{\cdot-}$ production in non-phagocytic cells is only about one-third that of neutrophils. $O_2^{\cdot-}$ and H_2O_2 are mainly produced intracellularly in vascular smooth muscle cells, in contrast to neutrophils, endothelial cells, and fibroblasts [4].

NOX1 expression is induced in various ways: in the vascular smooth muscle cells via the PDGF, PGF_2, and angiotensin II. It is increased after injury in the carotid artery and in the prostate after castration. Further conditions include the induction of epidermal growth factor and kinase activation, with NO causing a decrease. NOX3 is expressed in the inner ear; however, it is not yet entirely clear for what purpose constitutive production of ROS by NOX3 would be necessary in the inner ear. NOX4 is the constitutive enzyme, but its induction was observed in response to endoplasmic reticulum stress, the stress caused by pressure, arterial damage, hypoxia, and ischaemia, transforming growth factor (TGF-1), tumour necrosis factor (TNF), stimulation of smooth muscle as well as angiotensin II, insulin, and lipopolysaccharides. NOX5 is typical of the testis, spleen, lymph nodes, vascular smooth muscle, bone marrow, placenta, ovary, stomach, and is also present in several foetal tissues. It is not present in circulating lymphocytes and activation is controlled by Ca^{2+} concentration. Dual amino oxidases (DUOX) are NOX homologues, which act

like NOX2 single electron messengers and form $O_2^{\cdot-}$. They are particularly present in epithelial tissues and are induced by interleukins (IL-4, IL-13), DUOX2 interferon or directly by Ca^{2+} [29].

Turning xanthine oxidoreductase activity to oxidase: Xanthine oxidoreductase (XOR) exists in two mutually convertible forms as xanthine monoaminooxidase (XO) and xanthine dehydrogenase (XDH). XOR prevails in the form of XDH, and it is easily transformed into XO by oxidation of sulfhydryl groups or proteolysis [30]. The transition from XDH to XO seems to have a decisive influence on its physiological or pathological role in the development of disease states. For example, insufficient dismutation or overstimulation can result in the production of $O_2^{\cdot-}$ in the endothelium from the NOX enzyme. This can lead to oxidation of thiols in the XOR enzyme active site, changing it to the XO. Additionally, isoforms display different substrate affinity. XDH preferentially reduces NAD^+, whereas XO reduces O_2, and forms the superoxide radical and hydrogen peroxide [31].

In parallel reactions with the particles formed in other systems and from NOS activity, the oxidants are formed, and either will form other reactive intermediates in reaction with uric acid, causing oxidative tissue damage [32].

NOS uncoupling: Nitric oxide ($\cdot$NO) is characterized by the polymorphic effect and is the free radical itself. Its effects are predominantly intracellular. In the reaction of NO with $O_2^{\cdot-}$, a very strong oxidant is formed known as peroxynitrite anion (ONO_2^-). As part of the immune system, NO acts as effector and regulatory molecule. NO is responsible for the induction of the cytokines (IL-1 and IL-2), PGE_2 and other pro-inflammatory mediators. It is a major mediator in the cardiovascular system, mediating interactions between endothelial cells and smooth muscle cells, thereby substantially affecting vascular tone. It also serves as a mediator of central and peripheral neurons. The released NO performs two functions in the brain, both as an atypical neurotransmitter and in cell defence, due to its cytotoxic properties. In the peripheral nervous system, NO is a major neurotransmitter in the non-adrenergic and non-cholinergic neurons (mediating smooth muscle relaxation, e.g., in the gastrointestinal tract).Excessive production under pathological conditions may not only be involved in tissue damage, but can directly activate sensitive fibres in the CNS followed by the release of Calcitonin gene-related peptide (CGRP), thereby extending the burning pain [3,33].

NO is produced by three different isoforms of nitric oxide synthase (NOS). Neuronal (nNOS) and endothelial (eNOS) are constitutively expressed, and their activity is regulated by binding of Ca^{2+} to calmodulin (CaM). The inducible form (iNOS) is permanently CaM-bound, therefore the activity is not dependent on Ca^{2+}. It is regulated at the level of translation of various cytokines and bacterial toxins and their activity is not dependent on the concentration of Ca^{2+}. All three isoforms are functionally active as homodimers [34]. Reductase and oxygenase domains under certain circumstances are able to function as independent catalytic units; however, only the homodimer can perform the NO-synthase⁻ function.

In the absence of haem, NOS function as monomers [35]. However, monomers of all isoforms are incapable of binding tetrahydrobiopterine (BH4) without haem and do not catalyse the production of NO [35].

In the absence of BH4, nNOS and eNOS may form stable dimers which catalyse the simultaneous production of NO and $O_2^{\cdot-}$ [36,37], immediately resulting in ONO_2^- release. NOS only works as NO synthase at very high (up to 1 uM) concentrations of BH4 [38]. Under conditions of oxidative stress, however, it can very easily become scarce, as it acts as an effective radical scavenger. Its availability therefore depends on the sufficiency of ascorbate and glutathione [39]. Furthermore, upon a decrease in the concentration of L-arginine or its absence, NOS catalyses the uncoupled reduction of O_2, resulting in the production of $O_2^{\cdot-}$ and H_2O_2 [40]. Although $O_2^{\cdot-}$ is the initiator of other reactive species and the processes that lead to the creation of pathological conditions, the deleterious effects on endothelial function, endothelial smooth muscle cell proliferation and leukocyte adhesion observed in disease states are the result of ONO_2^- and the lack of NO.

It has been shown that uncoupled nNOS reaction is relevant in induced neuronal toxicity [41]. On the other hand, uncoupled iNOS is often present in a state where there is a lack of L-arginine, such as in wound repair. The eNOS uncoupled reaction is often due to lack of BH4 but not L-arginine [42].

Opioid receptors

Based on their affinity and intrinsic activity with the various opioid receptor subtypes, opioid analgesics are divided into pure agonists, partial agonists, antagonists, and mixed agonists-antagonists. Pure agonists elicit maximum biological response after binding to the receptor, activating receptors μ and partially κ receptors. Examples of a pure opioid receptor agonist are morphine, hydromorphone, oxycodone, pethidine, piritramide, fentanyl, sufentanyl. Partial agonists have a lower intrinsic activity in comparison with agonists, resulting in lower efficiency. A sub-maximal response is produced at higher doses. Partial agonists include, for example, buprenorphine. Mixed agonist-antagonist activity may differ at the different receptors. They may simultaneously act as an agonist at one receptor and antagonistic at the second. Pentazocine and nalbuphine are typical representatives of opioid receptor agonist-antagonists. Antagonists have zero intrinsic activity upon binding to the receptor.

This division is more or less theoretical. It is important to mention that pure agonists do not cause equally powerful effects at the various opioid receptors. It depends not only on its affinity (e.g. the dissociation constant) to the receptor, but also on the gender, race, existing conditions (hormonal balance), and so on. Rather, it is clear that each opioid acts on all opioid receptors at the same time, but with different intensity of induced effects. In some cases the response can have a sub-clinical effect. Also antagonists can trigger an agonistic effect on any opioid receptors, albeit slight and clinically insignificant [3,44].

023

The side effects of opioids

In practical terms, the side effects of opioids can be divided into temporary and long-lasting. Nervous system disorders manifested by the psychological state of the patient can cause confusion, mental and somatic dependency, dizziness and other symptoms. The autonomic nervous system may be affected and cardiovascular side effects may arise manifested by a fall in blood pressure, which is a consequence of vasodilation and decreased myocardial inotropic activity. Additional clinical signs are bradycardia characterized by general weakness, sweating, and pre-collapse and collapse states. The effects of opioids may cause respiratory depression, bronchospasm and bronchoconstriction.

Adverse effects on gastrointestinal tract lesions are manifested as nausea, vomiting, constipation, and, less frequently, dry mouth. Constipation does not develop tolerance and needs to be avoided (dietary modification, laxatives prevention), during long term treatment with opioids (by blocking peripheral opioid receptors in the gastrointestinal tract: application of an opioid antagonist methylnaltrexone, which does not cross the blood brain barrier, or use of naloxone, which is metabolized by "first-pass" metabolism in the liver, is an example of a combined preparation oxycodone/naloxone) [44]. Also disorders of parenchymatous organs especially the liver in relation to bottlenecks caused by biliary excretion of biliary tract spasm are known. Skin problems caused by the effects of opioids include urticaria, dermatitis, and pruritus. Renal and urinary disorders may develop and occur as urinary retention and ureteral spasm. Rarely, disorders of the immune system appear, which may lead to anaphylactic shock through the development of hypersensitivity [44].

Opioids have a negative effect on the endocrine system. Various studies have demonstrated the influence of regulatory mechanisms during opioid treatment. A fundamental change occurs at the hypothalamic-pituitary complex, which directs the activities of all endocrine systems. The secretion of hormones by the pituitary gland regulates the nervous system through the hypothalamus, which is the coordination centre of autonomic functions. The pituitary gland has a coordinating function in relation to other endocrine glands, thus the production of their hormones in turn affects the peripheral endocrine organs and then the target tissues [45].

Opioids decrease the secretion of gonadotropin-stimulating hormone, resulting in reduced levels of luteinizing hormone. The result is the reduced secretion of testosterone and estradiol, which in turn results in symptoms of hypogonadism. Chronic administration of exogenous opioids decreases the levels of adrenocorticotropic hormone and cortisol and the circadian rhythm. The result is a reduction in the response to stress. The effects of prolactin are not entirely clear. Opioids may stimulate thyroid stimulating hormone through the hypothalamus, which may cause a prolonged and enhanced response to opioids in patients with hypothyroidism. Chronic use of opioids is associated with weight gain, hyperglycemia and aggravate diabetes. This may be due to central effects through the sympathetic nervous system and impaired insulin secretion. New laboratory measurements show the development of oxidative stress in patients receiving morphine and related drugs [46].

Oxidative stress and opioids

Several studies show a correlation between the occurrence of oxidative stress and its subsequent complications, in treatment with opioids. The drug most suspected of exhibiting such side effects appears to be morphine. This effect is also likely from the semi-synthetic molecules derived from morphine, altered during degradation in the body to morphine or its metabolites. Other opioid studies have been sufficiently undertaken focussed on the excessive production of free radicals.

In principle, the production of reactive oxygen and nitrogen species in connection with long-term use of morphine can proceed in two possible pathways. The first are reactions following the activation of nitric oxide synthase (NOS); with regards this possibility, attention must be paid to the above points. The second pathway is the activation of the enzyme phospholipase A_2 (PLA_2). PLA_2 resides in the cytosol, translocates and binds to heparan sulphate proteoglycans on cell membranes in response to an increase in intracellular calcium [47,48]. With the proximity of membrane phospholipids, and prostanoid synthesis enzymes (COX and 5-LOX), arachidonic acid is released to couple COX [49]. PLA_2 is also internalized by neurons and contributes to increased eicosanoid synthesis and inflammatory pain [50]. However, during transactivation of iNOS involving NFκB, released NO interacts with COX-2 and influences prostanoid synthesis [51]. In order that that both pathways may be activated simultaneously, parallel stimulation can also take place through MAPK pathway and secondary formed peroxynitrite from iNOS activity. An increase in NOS activity as well as in the activities of the enzymes involved in the prostanoid synthesis are a significant source of reactive oxygen and nitrogen species. However, that is far from all when regarding expression in oxidative stress conditions. COX and LOX enzymes convert arachidonic acid only to prostanoid precursors PGG_2 and PGH_2 following conversion by tissue specific prostaglandin synthases to biologically active prostaglandins. Isomerisation to active prostanoids is achieved by the activity of MAPEG enzyme (membrane-associated proteins in eicosanoid and glutathione metabolism) (Jakobsson et al. 1999) working also as glutathione-S-transferase II in LTC4 production utilizing glutathione. Under inflammatory conditions, however, efficient antioxidant activity of glutathione would be more important, since oxidative stress limits the activity of the enzymes themselves involved in the synthesis of glutathione. In addition, glutathione serves as a reservoir for neuronal glutamate thus significantly affects neuronal sensation.

Studies assessing the issue of short-term and long-term effects of morphine showed a significant decrease in glutathione levels in brain tissue and liver of rodents as well as in human brain tissue. It was also found that chronic treatment with morphine reduced the activities of antioxidant enzymes

such as superoxide dismutase, catalase, glutathione peroxidase and other enzymes involved in antioxidant defence [52,53]. Many other internal and external factors are therefore involved in weakening the antioxidant activity in individuals burdened with opioids. This effect depends on the duration of treatment, the quantity of morphine, potential interactions with other drugs, and metabolic changes in various diseases. In general, the excessive production of reactive oxygen and nitrogen species, with simultaneous impairment of the elimination by antioxidant molecules, is associated with oxidative stress conditions. Interestingly, this effect was observed in both short- and long-term administration of morphine. A study on oxidative damage to the arteries revealed formation of reactive oxygen species following chronic administration of morphine in the endothelial cells of laboratory animals. This effect was also demonstrated later on human endothelial cells. This phenomenon can be explained by reduced nitric oxide concentration, followed by impaired vasodilation, and the subsequent development of atherosclerosis [54]. An increase in the concentration of hydrogen peroxide was observed in the prefrontal cortex and the striatum in rats administered with morphine. Increased concentrations of lipid peroxidation products in the hippocampus and liver were also measured [55].

Increased production of free radicals, especially superoxide, has been shown in tissue macrophages. In contrast, the results of experimental studies of human neuroblastoma cell lines SH-SY5Y have not shown prooxidative effects following morphine administration, nor accelerated the apoptosis of these cells. This evidence suggests an increased resistance of tumour tissue to the formation of free radicals and subsequent damage [56]. At the same time, it acknowledges the importance of taking into account the specific metabolism in different cell types, particularly following injury or during inflammatory processes accompanied by increased pain sensation. Tissue injury arising from ongoing exposure to high-intensity stimuli leads to a sensation of pain continuing beyond the removal of the originating stimulus. There is, in addition, an enhanced sensitivity to otherwise modestly aversive stimuli applied to the injured tissue (hyperalgesia) [57]. The high production of reactive oxygen and nitrogen species is then also the base for opioid tolerance development. These findings were confirmed through inhibition of NO and superoxide synthesis [58].

Reduction in ROS formation (especially NOS and NOX enzymes) results in a reduction in the likelihood of producing morphine-induced anti-nociceptive tolerance. This effect was demonstrated on the genetically modified NOS deficient mice, in which the chronic administration of morphine prevented the development of morphine-induced anti-nociceptive tolerance. Morphine induced activation of NADPH oxidase, which then catalysed the formation of superoxide and activates macrophages, this is one of several examples of the linking effect of morphine and its effect on the immune system. The neuroimmunological processes activated by the increased production of proinflammatory cytokines (TNF-α, IL-1β, IL-6) are equally important to consider. The signalling interaction is mediated through a μ-opioid receptor leading to the activation of PLA$_2$ and the subsequent cascade of biochemical events that lead to an increase in intracellular Ca^{2+}. The increased dose of morphine was found to increase expression of NMDA receptors. This phenomenon in the clinic can contribute to the creation of morphine tolerance and dependence. As Lamoria et al. [59] pointed out the reason is prolonged firing of C-fiber nociceptors during inflammation causing release of glutamate. The binding of glutamate to NMDA receptor causes opening of the ion channel portion of the receptor and influx of Ca^{2+}. The calcium induced changes in activation of pain signalling proteins involve phosphorylation of receptors, as well as changes in gene expression. After μ opioid receptor activation by morphine, signalling changes may occur leading to the removal of magnesium ion block of NMDA receptor. An opening the NMDA channel allows a calcium influx thus promoting the development of morphine tolerance. However, the use of a combination the NMDA-receptor antagonists with morphine might prevent secondary negative effect of morphine [60].

Oxidative stress resulting from morphine metabolism: Studies in several animal species have shown that morphine is metabolised by glucuronidation (in the 3- and 6- position) via UDP glucuronosyl transferase (UGT) and dealkylation [61]. UGT are membrane-bound microsomal enzymes. Glucuronidation reactions increase the polarity of xenobiotics, drugs and various endogenous substances. The carboxyl group of glucuronic acid is ionized at physiological pH, thus allowing higher solubility and easier elimination of glucuronides. These are then recognized by biliary and renal anionic transport system and eliminated in the urine and bile. Compared to other conjugation enzymes (acetyltransferases, glutathione-S-transferases, sulfotransferases), UGT have just a significant role in the formation of depositable metabolites through urine and bile. Glucuronidation modifies the structure of compounds as well as changing their metabolic activity, or cancelling it. In the case of generated morphine metabolites M-3-G and M-6-G, their biological activity increases [62].

UGTs are expressed in different tissues (including brain, prostate, uterus, placenta, kidney etc.) but the main location of glucuronidation is the liver. UGT genes form on the basis of sequence similarities the two gene families UGT1 and UGT2. Gene polymorphisms in UGT classes affect their physiological, clinical and pathophysiological effects [62-64]. UGT1 and 2, or isoforms, have not been greatly investigated so far, but they are possible targets for future study. It was found, for example, that polymorphisms in the UGT1A1 gene are risk factors for drug-induced toxicity. In a retrospective study of Iyer et al. [65] a link was found between alterations in the promoter and coding region of the gene UGT1A1 and severe irinoctan-induced toxicity in cancer patients. The specific alterations UGT genes contribute to the modification of risk factors for developing cancer. In Some subsequent studies have confirmed the importance of evaluating the UGT1A1 polymorphism before the initiation of chemotherapy [66,67].

Let us return to the more specific metabolism of morphine, which is up to 90% (ie usually) metabolised by UGT2B7. The study of Holthe et al. [68] noted that while the *2 allele

025

– substituting an amino acid at position 268 (Tyr268) – has similar catalytic properties to *1 allele, it is associated with changes in the ratios of morphine-3-G/morphine-6-G, morphine-3-G/morphine or morphine-6-G/morphine in plasma from cancer patients undergoing long term analgesic therapy with morphine. Regarding the decrease in morphine glucuronidation by UDP-glucuronosyltransferases (UGT), limited glucose metabolism may result from the effect of morphine as well as the disease itself. The liver is the site for metabolism of opioids. Some studies have confirmed that opioids affect the metabolism of glucose and the levels of hormone regulating glucose [69,70].

For example, in cancers, glucuronic acid is produced more from glycogen or glycogenic amino acids in the liver than from glucose. Therefore, the compounds to be metabolized by UGT cause a decrease in glycogen stores. The study by Favaro et al. [71], actually confirmed that, in cancer diseases, there is no mobilization of glycogen. Several factors may immediately be indicated that significantly affect and change the nature of redox processes:

1. UGT activity is limited because of the lack of glucuronic acid and accordingly the metabolism of administered drugs. Affects the redox state by restricting the formation of the largest source of NADPH in a cell in the pentose phosphate pathway – activity of glucose-6-phosphate dehydrogenase (G6PDH). It is known that the administration of morphine results in deactivation of the G6PDH. This happens by creating conjugates morphine-G6PDH [72].

That is why this second point seems to be more serious than the lack of glucuronic acid or alteration the UGT isoform activities, as morphine may be metabolised in two ways. The first pathway comprises known reactions:

morphine + $NADP^+$ (morphine dehydrogenase) → NADPH + morphinone

morphinone + NADH (morphinone reductase) → NAD^+ + hydromorphone

hydromorphone + NADPH (morphine dehydrogenase) → $NADP^+$ + dihydromorphone

However, NADPH is also required for the activities of:

– NADPH oxidases, since their activity is particularly pronounced in cells of the immune system during inflammation. However, as glutamine is used for its creation, the production of reactive oxygen species and superoxide anion radicals is not affected.

– NOS. Sufficiency of cofactors and substrates significantly affect the activity of all three isoforms of NOS. In the absence of NADPH, tetrahydrobiopterin, and the substrate arginine (or even lack of it) leads to the uncoupling of NOS activity, while at the same time NO and superoxide are formed. Species immediately react to form peroxynitrite [37,73], resulting in a lack of NO. Pterin acts as a potent scavenger of $O_2^{\cdot-}$, thus preventing

rapid reaction of $O_2^{\cdot-}$ with NO and releases it in the form of H_2O_2. Only in the absence of haem are NOS not able to bind tetrahydrobiotperine and catalyse the formation of NO [35].

– Glutathione reductase. Its role is to return oxidized glutathione (GSSG) to reduced (GSH) through NADPH reduction.

The second route of metabolism is mediated through cytochromes.

Morphine, oxymorphine, and hydromorphone can be metabolized by N-demethylation by cytochrome P450 (CYP3A4 and CYP2C8). CYT are haemoproteins whose reaction mechanism also depends on NADPH, using electrons to generate reduced haem. The reduced haem allows binding of O_2 and the oxidation of the substrate by the insertion of one oxygen to a substrate and second to the molecule of water. The reaction may be illustrated as follows: P450red + RH + O_2 → P450ox + ROH + H_2O. Perferyl complex $[Fe^{5+} = O]^{3+}$, which is formed in the haem after taking the electron, is able to bind only H instead of OH group, thus forming alkyl radicals. Radicals damage cell structures by reactions with proteins to form stable adducts. In addition, singlet oxygen can be formed as by-products, and reactions are even capable of reducing molecular oxygen to superoxide and/or H_2O_2 [4].

Furthermore, a unique feature of NOS has significant influence. The enzyme is similar to cytochrome P450 and catalyses other oxidizing reactions, which also form radical intermediates. The mechanism of oxidation of substances is explained by the mechanism of cytochrome P450 [74]. The fact that NOS metabolises and affects the efficiency and tolerance to morphine has also been suggested by some studies. For example, through the effects of arginine as described by Karami et al., Barghava et al. [75,76], found that the anti-nociceptive effect of morphine as well as its concentration reduced the administration of L-arginine. In contrast, D-arginine administration, did not show this effect. This clearly indicates a significant effect of NOS on the metabolism, the effectiveness of morphine and the generation of reactive species, as NOS has a limited substrate specificity and is unable to use D-arginine for NO synthesis [74].

Conclusion

The fact that oxidative stress plays a key role in the pathogenesis of many diseases is a generally accepted conclusion from experimental research. Subsequently, therapeutic options were also adjusted. New clues contribute to the finding that the acceleration of oxidant states, as well as the weakening of the redox capacity of the internal environment, may also participate the long-term use of opioids. Most published papers covered the pro-oxidant activity of morphine while the effects of other opioids for the treatment of severe pain have yet to be significantly studied. The conclusions of this study could clarify the hypotheses and the subsequent possibility of targeted antioxidant intervention. For clarification of the evidence of the intensity of oxidative stress in patients with

026

chronic pain treated with opioids for severe pain, we are performing a prospective observational multicenter study the results of which will be the subject of experimental study.

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