CHAPTER 6

Melanocortins and the Cholinergic Anti-Inflammatory Pathway

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Abstract

Experimental evidence indicates that small concentrations of inflammatory molecules produced by damaged tissues activate afferent signals through ascending vagus nerve fibers, that act as the sensory arm of an "inflammatory reflex". The subsequent activation of vagal efferent fibers, which represent the motor arm of the inflammatory reflex, rapidly leads to acetylcholine release in organs of the reticuloendothelial system. Acetylcholine interacts with $\alpha 7$ subunit-containing nicotinic receptors in tissue macrophages and other immune cells and rapidly inhibits the synthesis/release of tumor necrosis factor-α and other inflammatory cytokines. This neural anti-inflammatory response called "cholinergic anti-inflammatory pathway" is fast and integrated through the central nervous system. Preclinical studies are in progress, with the aim to develop therapeutic agents able to activate the cholinergic anti-inflammatory pathway. Melanocortin peptides bearing the adrenocorticotropin/α-melanocyte-stimulating hormone sequences exert a protective and life-saving effect in animals and humans in conditions of circulatory shock. These neuropeptides are likewise protective in other severe hypoxic conditions, such as prolonged respiratory arrest, myocardial ischemia, renal ischemia and ischemic stroke, as well as in experimental heart transplantation. Moreover, experimental evidence indicates that melanocortins reverse circulatory shock, prevent myocardial ischemia/reperfusion damage and exert neuroprotection against ischemic stroke through activation of the cholinergic anti-inflammatory pathway. This action occurs via stimulation of brain melanocortin MC_3/MC_4 receptors. Investigations that determine the molecular mechanisms of the cholinergic anti-inflammatory pathway activation could help design of superselective activators of this pathway.

Introduction

Melanocortins have long been considered to only exert control on endocrine and pigmentary processes. For the first time our Teacher, Professor William Ferrari, in 1955,[1-3] then Professor David De Wied[4] and over the subsequent decades several other independent groups, reported many important extra-hormonal effects of melanocortins.[5-10] In addition to the pituitary gland, production of melanocortins was also documented in a variety of peripheral tissues and within the central nervous system (CNS).[5,9,11] Altered production of these neuropeptides has been recognized among the causes of many morbid conditions including anorexia, hyperfagia, obesity, cachexia, pain, inflammation, sexual dysfunctions, circulatory shock, organ damage induced by ischemia/reperfusion, neurodegeneration.[5,7,9-13] α-Melanocyte-stimulating hormone (α-MSH) is the natural

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melanocortin peptide able to induce all extra-hormonal effects of melanocortins. Identification and cloning of the five melanocortin receptors (MC_1-MC_5) in the nineties,[14-21] as well as the synthesis of selective agonists and antagonists at MC receptors,[13,16,21] increased the general interest for this family of molecules in view of their possible therapeutic use.[5,7,11,13,22-25]

The protective and resuscitating effects of melanocortins in extreme hypoxic condition appear particularly promising for therapeutic purposes. Adrenocorticotropin (ACTH)/α-MSH sequences, as well as shorter fragments and synthetic analogs, have a life-saving effect in animals and humans in conditions of circulatory shock.[26-34] These neuropeptides are likewise protective in other severe hypoxic conditions, including prolonged respiratory arrest,[35] myocardial ischemia,[36-41] renal ischemia[42-44] and ischemic stroke,[45-49] as well as in experimental heart transplantation.[50]

Preclinical evidence indicates that melanocortins produce their protective and life-saving effects, at least in part, by activating the recently recognized "cholinergic anti-inflammatory pathway", the motor arm of the "inflammatory reflex".[51-53]

The Inflammatory Reflex

In the past, the vagus nerve was only considered a part of the parasympathetic nervous system with precise functions, such as the regulation of heart and gut activities and respiration. However, more recent data have disclosed previously unrecognized functions of the vagus nerve. Electrical stimulation of the vagus nerve is currently approved for treatment of epilepsy and major depression; additional therapeutic uses under investigations include obesity, Alzheimer's disease, chronic pain and some neuropsychiatric disorders.[54,55] The mechanisms underlying the beneficial effects of the vagus nerve therapy are not fully understood.

The CNS modulates local and systemic inflammatory responses to various stressor agents through humoral and neural mechanisms.[56-58] High levels of cytokines and other inflammatory mediators can reach brain areas devoid of blood-brain barrier (dorsal vagal complex including the sensory nuclei of the solitary tract, area postrema and dorsal motor nucleus of the vagus).[59-61] This humoral route for communication between the immune system and the CNS seems to be involved in several processes, including fever, anorexia and activation of hypothalamic-pituitary responses. Knowledge of the humoral route was the base for development of anti-inflammatory drugs including glucocorticoids.[52,62-64]

Efferent vagus nerve signalling contributes to modulation of inflammation. Efferent vagal signalling may facilitate lymphocyte release from the thymus through a nicotinic acetylcholine receptor-mediated mechanism; moreover, clinical studies indicate that nicotine exerts beneficial influences in inflammatory bowel disease.[52] These observations led Tracey and coworkers to hypothesize that the parasympathetic nervous system could modulate the systemic inflammatory response, as an alternative mechanism for rapid cytokine control. They verified this hypothesis in experimental models of endotoxic shock in rats and their investigations led to the identification of the "cholinergic anti-inflammatory pathway", the motor arm of the "inflammatory reflex".[51] Low levels of inflammatory molecules produced in damaged tissues activate afferent signals through ascending vagus nerve fibers (neural inflammation-sensing pathways at low threshold of detection) and this could serve as the sensory arm of the inflammatory reflex.[52,62-64] The subsequent activation of vagus efferent activity, which includes the motor arm of the inflammatory reflex, rapidly leads to acetylcholine release in organs of the reticuloendothelial system (liver, lung, heart, spleen, kidney and gastrointestinal tract). Acetylcholine interacts with α7 subunit-containing nicotinic acetylcholine receptors in tissue macrophages and other immune cells surrounding the cholinergic terminals and rapidly inhibits synthesis/release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and other cytokines: this neural anti-inflammatory response is fast and integrated within the CNS.[51-53,65] The old observations by Guarini and coworkers,[66,67] that nicotine and dimethylphenylpiperazinium reverse hemorrhagic shock through a peripheral, nicotinic receptor-mediated mechanism, can be explained on the basis of these novel findings.

Based on the observations by Tracey and coworkers, our group identified the cholinergic anti-inflammatory pathway in hemorrhage-shocked rats.[65] Subsequently, several other groups

have shown the activity of this cholinergic anti-inflammatory pathway in various experimental conditions characterized by a systemic inflammatory response.[52,53,68-71] Preclinical studies are in progress to verify the role of the cholinergic anti-inflammatory pathway in local and systemic experimental diseases. Indeed, activation of this pathway could be used therapeutically. Vagus nerve control of visceral organs is integrated and regulated through the CNS. Reciprocal neural interconnections among the nucleus tractus solitarius, dorsal motor nucleus of the vagus, nucleus ambiguus, other forebrain structures such as hypothalamus, amygdala and insular cortex, form a brain network that regulates efferent vagal activity and related visceral organ functions.[72] Receptors belonging to this central autonomic network could be targets for activators of the cholinergic anti-inflammatory pathway.

Antishock Effects of Melanocortins

Circulatory shock is a severe pathological condition accompanied by a systemic inflammatory response, with upregulated expression of inflammatory mediators and recruitment of inflammatory cells in several tissues. Activation of nuclear transcription factors including NF-kB triggers an inflammatory cascade with production of cytokines, such as TNF-α, chemokines, cell adhesion molecules, free radicals including nitric oxide and other inflammatory mediators.[31,32,34,51-53,65,73-77] The multiple organ injury that is often associated with shock can be caused by both hypoperfusion and reperfusion during resuscitation.[73,78-80]

The antishock effects of melanocortin peptides ACTH-(4-10), α-MSH, ACTH-(1-17), ACTH-(1-24), discovered in our laboratory, has been subsequently confirmed in several studies. The original investigations were performed in a severe model of hemorrhagic shock, induced by stepwise withdrawal of about 50% of the circulating blood in rats and dogs. This procedure causes death in all animals within 30-35 min.[26,27,81-83] Conversely, intravenous injection of melanocortin peptides rapidly induces a dose-dependent restoration of arterial blood pressure and tissue blood flow. Normalization of arterial and venous pH and base excess, as well as of venous tension of O_2 and CO_2 and of venous oxygen saturation and lactate, also gradually occur.[84] The reversal of hemorrhagic shock induced by melanocortin peptides is associated with marked increase in circulating blood volume. Such increase is the consequence of mobilization of the peripherally pooled residual blood from the liver, spleen and other organs.[28,83,85] Subsequent studies using the same experimental model indicated that melanocortins greatly prolong survival and extend the time-limit for effective blood reinfusion (up to 3-4 hours after shock, versus 10-15 min in saline-treated animals) for complete shock reversal.[29]

These resuscitating effects have been confirmed in the same animal models of hemorrhagic shock,[86] as well as in hemorrhage-shocked/resuscitated hamsters,[87] in hypovolemic shock induced in rabbits by graded occlusion of the inferior vena cava,[88] in a rat model of splanchnic artery occlusion[34] and in a severe model of prolonged respiratory arrest in rats.[35] ACTH-(1-24) has then been successfully used in human conditions of hemorrhagic and cardiogenic shock (intravenous bolus of 5-10 mg), both in anecdotal cases and randomized controlled studies.[33,89-92]

Clinical studies on the effectiveness of melanocortins in circulatory shock are in progress. From a practical point of view, availability of drugs able to retard shock progression toward an irreversible state, extending the time-limit for a successful first aid of civilian and military victims of traumatic accidents, is of great importance.

The antishock effects of melanocortins are adrenal-independent, can be obtained with intracerebroventricular injection of doses much lower than those required by the intravenous route and are mediated by melanocortin MC_4 receptors located in the CNS.[26,30,32,82] Such effects are associated with marked reduction in NF-κB activation and plasma concentrations of inflammatory mediators, including TNF-α, oxygen free radicals and nitric oxide and in intercellular adhesion molecule expression by vascular endothelium.[31,34,75,76,93] This action is consistent with the established anti-inflammatory influence of melanocortins[7,11] and suggests that the ability of these neuropeptides to extend the time-limit for an effective blood reinfusion[29] may be due to blockade of the mechanisms responsible for late organ failure and death.

The antishock effect of melanocortins is prevented by a) bilateral cervical vagotomy, b) the intracerebroventricular injection of the acetylcholine-depleting agent hemicholinium-3 and c) the pharmacological blockade of central (but not peripheral) muscarinic acetylcholine receptors.[94,95] These observations suggest a contribution by CNS cholinergic pathways involving muscarinic receptors. Indeed, cholinomimetic agents, able to cross the blood-brain barrier, likewise reverse hemorrhagic shock, in rats and rabbits.[96-98]

The impressive resuscitating effect of melanocortins in severe shock models, the ineffectiveness of the conventional antishock drugs in the same animal models,[99] as well as the beneficial results obtained in human conditions of hemorrhagic and cardiogenic shock, suggest that melanocortins acting at MC_4 receptors could be innovative and promising resuscitating drugs in conditions of circulatory shock.

Melanocortins Reverse Circulatory Shock through Activation of the Cholinergic Anti-Inflammatory Pathway

Efferent vagus nerve signalling reduces the systemic inflammatory response in endotoxic shock. Indeed, electrical stimulation of efferent vagal fibers during experimental lethal endotoxemia blunts hepatic TNF-α synthesis/release, attenuates serum levels of this cytokine and prevents shock development.[51] Moreover, acetylcholine reduces release of several inflammatory cytokines by lipopolysaccharide-stimulated human macrophages and this effect is counteracted by nicotinic receptor antagonists. In hemorrhagic shock, as well as in splanchnic artery occlusion shock, electrical stimulation of efferent vagal fibers rapidly reverses hypotension, prevents hepatic NF-κB activation, blunts hepatic TNF-α synthesis/release, lowers TNF-α serum levels and improves survival in rats.[65,77] These findings suggest that a "cholinergic anti-inflammatory pathway" operates during endotoxic, hemorrhagic and splanchnic artery occlusion shock, to counterbalance development of the inflammatory cascade responsible for vascular derangement and multiple organ failure (Fig. 1). Identification of drugs able to activate this pathway might provide highly effective and innovative approaches for treatment of circulatory shock.

As reviewed above, melanocortin peptides of the ACTH/MSH family exert a prompt and sustained resuscitating effect in conditions of circulatory shock. This effect is adrenal-independent and occurs through inhibition of the systemic inflammatory response.[31,34,75,76,93] Previous investigations on the anti-inflammatory effects of melanocortins showed that systemic administration of α-MSH reduces blood concentrations of IL-1α and TNF-α in a mouse model of lipopolysaccharide-induced systemic inflammation.[100] Furthermore, Lipton and coworkers showed that in a mouse model of peritonitis/endotoxemia induced by cecal ligation and puncture, systemic treatment with α-MSH improves survival.[101] Collectively, these findings indicate that melanocortins counteract the systemic inflammatory response in circulatory shock.

The next question was whether hemorrhagic shock reversal produced by melanocortins depends on activation of the vagus nerve-mediated cholinergic anti-inflammatory pathway.[31] Action potential recordings in hemorrhage-shocked rats treated with nanomolar concentrations of ACTH-(1-24), indicate that neural efferent activity along the vagus nerve markedly increases. This effect is associated with the restoration of cardiovascular and respiratory functions, blunted NF-κB activity and decreased TNF-α in liver and plasma. Bilateral cervical vagotomy, or pharmacological blockade of brain melanocortin MC_4 receptors and muscarinic acetylcholine receptors, or of peripheral nicotinic acetylcholine receptors, prevents the life-saving effect of ACTH-(1-24) and the associated effects on NF-κB activity and TNF-α levels.[31] Blockade of brain MC_4 receptors and muscarinic receptors (a) blunts the stimulating effect of ACTH-(1-24) on efferent vagal activity, (b) reduces, like bilateral cervical vagotomy, the blood volume to be withdrawn in order to induce shock and (c) prevents the compensatory increase in efferent vagal activity normally occurring during bleeding in control shocked animals.[31]

Collectively, these findings indicate that melanocortins suppress the NF-kB-dependent systemic inflammatory response triggered by hemorrhage and reverse shock condition, through activation of the cholinergic anti-inflammatory pathway within the brain (Fig. 1). These results likewise indicate
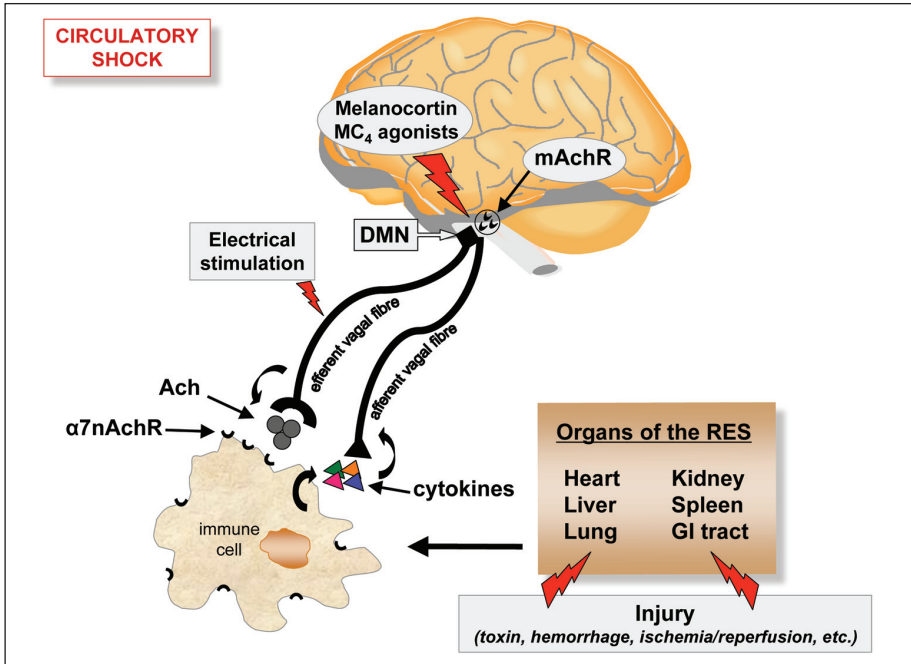
Figure 1. Modulation of the inflammatory response in circulatory shock, through the cholinergic anti-inflammatory pathway. Low levels of inflammatory molecules produced in damaged tissues activate afferent signals through ascending vagus nerve fibers (sensory arm of the inflammatory reflex). Activation of the cholinergic anti-inflammatory pathway (motor arm of the inflammatory reflex, that is impaired during shock) by electrical stimulation of efferent vagal fibers, or by stimulation of brain melanocortin MC_4 receptors (likely in the vagus dorsal motor nucleus: DMN), rapidly leads to acetylcholine (Ach) release in organs of the reticuloendothelial system (RES). Acetylcholine released from efferent vagal terminals interacts with α7 subunit-containing nicotinic acetylcholine receptors (α7nAchR) on tissue macrophages and other immune cells surrounding the cholinergic terminals and inhibits the synthesis/release of inflammatory cytokines, with consequent reduction in cytokine plasma levels and attenuation of the systemic inflammatory response. Acetylcholine interaction with peripheral muscarinic receptors (mAchR) does not play a role in circulatory shock. On the contrary, brain muscarinic receptors also are involved in triggering the cholinergic anti-inflammatory pathway.

that blockade of either brain MC_4 receptors, or brain muscarinic receptors, or efferent vagal transmission, accelerates the evolution of shock. These observations suggest the existence of a melanocortin-dependent antishock pathway. The involvement of brain muscarinic receptors in activation of the cholinergic anti-inflammatory pathway has been subsequently confirmed in experimental endotoxic shock.[102] Blockade of the cholinergic anti-inflammatory pathway activity by the nicotinic acetylcholine receptor antagonist chlorisondamine provides a mechanism for the original observations by Guarini and coworkers,[66,67] that dimethylphenylpiperazinium and surprisingly nicotine (a CNS acting drug) reverse hemorrhagic shock through a peripheral, nicotinic receptor-mediated pathway.

Interestingly, melanocortins exert their well-known anti-inflammatory activity mainly via a central mechanism that leads to reduced pro-inflammatory cytokines and chemokines production and increased release of the anti-inflammatory cytokine IL-10.[11] This mechanism partly overlaps with the cholinergic anti-inflammatory pathway, which modulates the systemic inflammatory response in shock conditions by inhibiting monocyte production and release of pro-inflammatory cytokines, but not of the anti-inflammatory cytokine IL-10.[51]

Protective Effect of Melanocortins in Myocardial Ischemia/Reperfusion

Melanocortins are also highly effective in treatment of animal models of myocardial ischemia. Ischemia rapidly causes profound biochemical and morphological changes and induces an inflammatory reaction in the heart tissue. Reperfusion is associated with severe alterations of cellular metabolism that may lead to further tissue injury. Myocardial reperfusion causes severe arrhythmias, endothelial dysfunction, myocardial stunning, cell death either by necrosis or apoptosis and a high lethality.[36,103,104] It appears that myocardial ischemia triggers apoptosis and reperfusion accelerates the process.[105] Both ischemia and reperfusion induce oxidative and nitrosative stress[36,106-108] as well as early activation of mitogen-activated protein kinases and NF-κB[109,110] in cardiac myocytes. These signal transduction mechanisms may in part contribute to cardiac injury, by causing early increase in expression of heart damaging factors and in part to cardioprotection.[111] A number of innovative pharmacological approaches to myocardial ischemia/reperfusion injury have been investigated, but the results are either conflicting or not confirmed in clinical trials.[105,112-114]

We have shown that melanocortins, including $[Nle^4,D-Phe^7]\alpha$-MSH (NDP-α-MSH), γ_1-MSH and γ_2-MSH, injected intravenously during experimental coronary occlusion, exert a protective effect both in rats subjected to transient myocardial ischemia followed by reperfusion—an animal model characterized by high incidence of ventricular tachycardia (VT), ventricular fibrillation (VF) and death—and in rats subjected to permanent coronary artery occlusion.[36,37,39,115] Myocardial ischemia/reperfusion injury also activates the anti-apoptotic, pro-survival cascades of the phosphatidylinositol 3-kinase-Akt and extracellular signal-regulated kinases (ERK 1/2), that appear to make up an universal pro-survival signalling pathway mediating myocardial protection at reperfusion.[111] In the rat model of myocardial ischemia/reperfusion described above, ACTH-(1-24) enhanced ERK 1/2 activation, triggering therefore the pro-survival cascade. In addition, melanocortins reduced histological alterations in the left ventricle, including those involving structural proteins, counteracted the inflammatory response and stimulated anti-apoptotic reactions.[40]

The cardioprotective effect of melanocortins has been confirmed in rat hearts isolated 12 hours after treatment with α-MSH or ACTH-(4-10) and then subjected to a 30-min period of ischemia followed by 120 min of reperfusion.[41,116] In this experimental model, melanocortin treatment results in a reduction in VF, infarct size and activity of the apoptotic protein caspase-3 and in an increase in the expression of the anti-inflammatory protein heme oxygenase-1. Melanocortins have also been shown to be effective in mouse models of myocardial ischemia/reperfusion.[12,38]

The prevention of ventricular arrhythmias in transient myocardial ischemia and the reduction of infarct size in permanent ischemia may be due to the melanocortin ability to inhibit the oxygen free radical discharge and to reduce the inflammatory and apoptotic responses.[12,36,39,40] Indeed, melanocortin peptides have a peculiar, adrenal-independent, anti-inflammatory activity.[7,11,21] An anti-apoptotic activity, has also been demonstrated.[5,22,45]

It appears that the cardioprotective effect of melanocortins could be mediated by brain melanocortin MC_3 receptors.[36,37,39,40,115] Indeed, when the selective MC_3 agonist γ_2-MSH was administered intracerebroventricularly, a dose ten times lower than that needed by the intravenous route provided full protection. This observation could explain the reason why treatment of isolated rat hearts with ACTH-(4-10) given at the time of reperfusion, rather than "in vivo" before heart harvest, failed to improve the post-ischemic recovery.[116] However, the data of Getting and coworkers[38] suggest a minor participation of cardiac MC_3 receptors.

Interestingly, melanocortins also cause a significant increase in allograft survival in experimental heart transplantation. Although they do not eventually prevent rejection, treatment was associated with a marked decrease in leukocyte infiltration and in expression of inflammatory molecules involved in allograft rejection.[50] Gene expression profile studies have revealed that melanocortin treatment of recipients preserves transplanted heart function by altering multiple protective pathways.[117]

Thus, melanocortins could be an useful tool for the prevention of cardiac damage, in different conditions of ischemia/reperfusion injury.

Melanocortins Prevent Myocardial Ischemia/Reperfusion-Induced Damage through Activation of the Cholinergic Anti-Inflammatory Pathway

Electrical stimulation of the vagus nerve has been proposed as a novel approach for treatment of myocardial ischemia/reperfusion injury. Indeed, stimulation of the vagus nerve prevented VF and VT in cats after myocardial reperfusion following a 20-min period of coronary occlusion[118] and VF in dogs with a healed myocardial infarction.[119] Moreover, it has been reported that vagus stimulation improves long-term survival after chronic heart failure in rats.[120] These investigations have provided a robust framework for the interpretation of findings by Cheng and coworkers.[121]

The nucleus ambiguus has long been considered the major vagal nucleus controlling the heart activity, whereas the vagus dorsal motor nucleus was thought to play only a marginal role. Recently, Cheng and colleagues[121] provided anatomical evidence for dual vagal cardiac efferent pathways in rats, that could play different roles in control of heart function. The data showed that neurons of the nucleus ambiguus and dorsal motor nucleus of the vagus project axons that converge to the same cardiac ganglia and innervate separate nonoverlapping populations of principal neurons within each cardiac ganglion. These findings, together with functional observations by the same investigators,[122,123] suggest that both brain nuclei play important, though different, roles in controlling cardiac function. During myocardial ischemia and reperfusion, therefore, one of these dual vagal cardiac efferent pathways could be a cholinergic anti-inflammatory pathway, involved in cardioprotection, as it occurs in circulatory shock. Indeed, in conditions of heart failure, a systemic inflammatory response plays a fundamental pathogenetic role.[124]

These observations encouraged further investigations, aimed at determining whether melanocortin peptides activate such vagal efferent pathway(s) in experimental conditions of ischemic heart disease. In rats subjected to coronary artery occlusion (5 min) followed by reperfusion (5 min), electrical stimulation of efferent vagal fibers (5 V, 2 ms, 1-9 Hz, for the whole period of ischemia/reperfusion) has been shown to strongly reduce the incidence of severe arrhythmias and lethality and the increases in free radical blood levels and left ventricle histological alterations and to augment the activation of the anti-apoptotic prosurvival kinase ERK 1/2.[40] Nanomolar amounts of the melanocortins ACTH-(1-24) (agonist at all MC receptors) and γ_2-MSH (selective agonist at MC_3 receptors)—administered in rats during coronary occlusion, intravenously or intracerebroventricularly at a dose 10 times lower—produces the same protective effects of efferent vagal fiber electrical stimulation (Fig. 2).[40] Since bilateral cervical vagotomy blunts the beneficial effect of ACTH-(1-24) and of the selective MC_3 agonist γ_2-MSH, the protective effect of melanocortins likely involves such vagal pathway. Accordingly, blockade of peripheral muscarinic acetylcholine receptors prevents the effects of both electrical stimulation and melanocortins; in the latter case, also central muscarinic receptors seem to be involved.[40]

More recently, the improvement in functional recovery, following vagus nerve electrical stimulation of isolated rat hearts subjected to ischemia/reperfusion injury, has been associated with inhibition of cardiomyocyte adenosine triphosphate depletion; indeed, it is established that rapid depletion of adenosine triphosphate leads cells to death. In the same investigation, the beneficial effect of acetylcholine against depletion of adenosine triphosphate has been also shown in isolated cardiomyocytes subjected to hypoxia/reoxygenation. In both studies, the protective effects seem to be mediated by muscarinic acetylcholine receptors.[125]

Overall, these results indicate a protective, efferent vagal cholinergic pathway operative in conditions of ischemic heart disease, that could be activated by melanocortins (Fig. 2). The melanococortin-induced activation of such a pathway seems to be triggered by stimulation of brain MC_3 receptors,[37,40,115] with involvement of brain and (as main final step) heart muscarinic receptors. Ischemia/reperfusion injury is still considered a major problem in patients with acute coronary occlusion and in patients undergoing surgical operations, such as coronary artery bypass grafting. These patients could benefit by a pharmacological activation of the cholinergic anti-inflammatory pathway. Benefits for patients with ischemic heart diseases by melanocortin-induced triggering
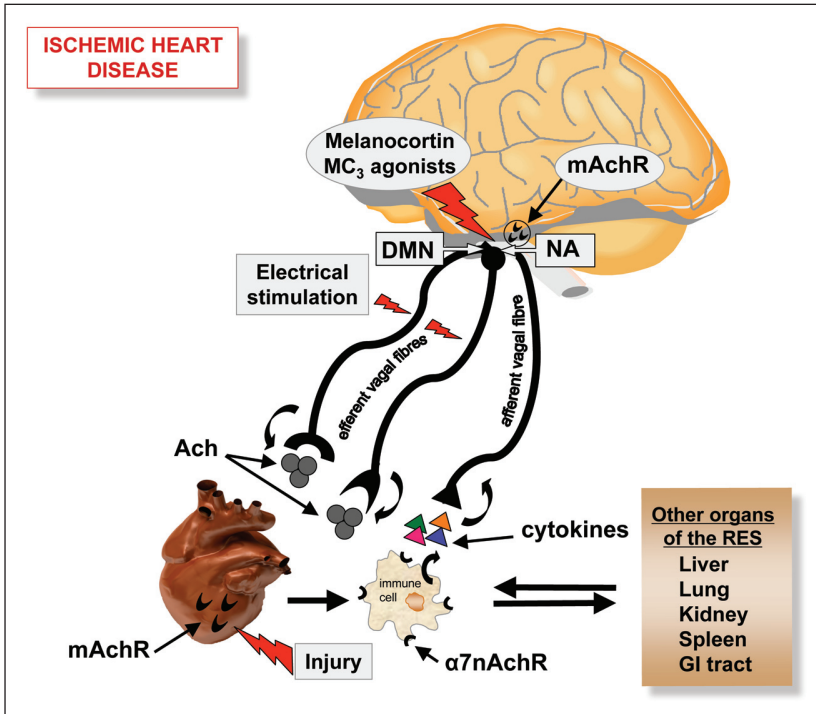
Figure 2. Modulation of the inflammatory response in myocardial ischemia/reperfusion and heart failure, through the cholinergic anti-inflammatory pathway. Low levels of inflammatory molecules produced in damaged tissues activate afferent signals through ascending vagus nerve fibers (sensory arm of the inflammatory reflex). Activation of the cholinergic anti-inflammatory pathway (motor arm of the inflammatory reflex, that is impaired during ischemia/reperfusion) by electrical stimulation of efferent vagal fibers, or by stimulation of brain melanocortin MC_3 receptors likely located in the vagus dorsal motor nucleus (DMN) or nucleus ambiguous (NA), rapidly leads to acetylcholine (Ach) release in the heart. Acetylcholine released from efferent vagal terminals interacts with muscarinic acetylcholine receptors (mAchR) on myocardial tissue leading to cardioprotection. Likely, acetylcholine interaction with peripheral nicotinic receptors (α7nAchR) in organs of the reticuloendothelial system (RES) plays a role in myocardial ischemia/reperfusion and heart failure, a condition where also a systemic inflammatory response can occur. Brain muscarinic receptors are also involved in triggering the cholinergic anti-inflammatory pathway.

of the cholinergic anti-inflammatory pathway could be even greater, because of the established ability of melanocortins to increase the anti-inflammatory cytokine IL-10 production.[11] Indeed, modulation of inflammation with IL-10 attenuates left ventricle disfunction and remodeling after acute myocardial infarction in mice.[126]

Neuroprotection by Melanocortins in Ischemic Stroke

Stroke is the third main cause of death and the leading cause of adult disability in developed countries. Within minutes to days after a cerebral vessel occlusion, several pathological pathways are triggered, which may potentially damage brain cells.[25,127] Despite intensive investigations aimed at developing innovative neuroprotective treatments for brain injury, no novel drugs have established clinical effectiveness. Toxic side effects, short therapeutic treatment window, single-mechanism of neuronal damage blockade are the main problems.[127-129] Indeed, information from clinical trials

suggests that targeting an array of key pathophysiological mechanisms is essential for an effective and safe management of stroke.[25,127,128,130] At present, the only approved therapy for ischemic stroke is thrombolysis within 3 hours of symptom onset.[25,128,131] However, only 3% of all stroke patients can receive thrombolytic agents, only few of treated patients experience some benefits and a growing body of evidence indicates that these drugs also have deleterious effects.[25,132]

Protective properties of melanocortins in experimental brain ischemia have been reported by Lipton's group. These experiments showed that α-MSH improves recovery of auditory-evoked potentials in a dog model of transient brain stem ischemia.[133] Tatro's group reported that α-MSH reduces brain TNF-α levels in transient global and focal cerebral ischemia in mice.[134] Recently, our research group provided the first clear evidence that melanocortins afford a strong neuroprotection against damage consequent to global or focal cerebral ischemia in gerbils and rats, through the activation of CNS melanocortin MC_4 receptors.[45-47,49] Eleven-day intraperitoneal treatment with nanomolar amounts of the melanocortin NDP-α-MSH, but not of the selective MC_3 receptor agonist $γ_2$-MSH, protects against impairment in learning and memory caused by transient global brain ischemia in gerbils (induced by occlusion of the common carotid arteries for 10 min).[45,46] This protective and long-lasting (67 days, at least) effect, which is prevented by pharmacological blockade of MC_4 receptors, occurs also when treatment starts several hours after ischemia (being 18 hours the approximate time-limit). In transient global brain ischemia in gerbils, functional recovery after stroke is associated with a modulation of the excitotoxic, inflammatory and apoptotic responses in the hippocampus and with a consequent reduction of the morphological damage and an increase in the number of viable neurons. The same melanocortin treatment given for 11 days also protects against impairment in learning and memory, sensory-motor orientation and coordinated limb use in a rat model of focal cerebral ischemia caused by intrastriatal microinjection of endothelin-1.[47] In this severe experimental model, the MC_4 receptor-dependent protective effect of NDP-α-MSH is associated with diminished excitotoxic, inflammatory and apoptotic reactions.[49] Other beneficial features involve a significant reduction of the severe morphological damage of the nucleus striatum, including a reduction of neuronal death, demyelination and phagocytic activity. Neuroprotection is also associated with a significant increase in number of small vessels within ischemic areas, relative to saline-treated rats. Of great importance, also in these rat studies NDP-α-MSH showed a broad therapeutic treatment window.[47] Subsequently, Forslin Aronsson and coworkers[135] and Chen and coworkers[48] confirmed the neuroprotective effect of α-MSH in other models of global and focal cerebral ischemia, without investigating MC receptors.

In conclusion, melanocortin agonists at MC_4 receptors appear to produce effective neuroprotection with a broad time window and through counteraction of the main ischemia-related mechanisms of brain damage. Furthermore, the antipyretic action of melanocortins is well established[136] and hypothermia might contribute to neuroprotection during cerebral ischemia.[137] Stroke therapy with these neuropeptides, therefore, could take a further advantage by melanocortin-induced hypothermia.[25] No novel drug has so far been shown to possess so many favourable and promising characteristics, at least in experimental stroke models.

Melanocortins Produce Neuroprotection Against Ischemic Stroke by Activating the Cholinergic Anti-Inflammatory Pathway

Observations in animals[138,139] and humans[140,141] indicate that stroke is associated with systemic pathophysiological reactions. Widespread production/activation of inflammatory mediators in the peripheral immune system following focal cerebral ischemia has been reported in mice.[142]

The systemic pathophysiological reactions associated with focal cerebral ischemia and a possible protective involvement of the cholinergic anti-inflammatory pathway, have been investigated in rats.[49] Following intrastriatal microinjection of endothelin-1, the activation of ERK 1/2, c-jun N-terminal kinases and caspase-3, the increase in TNF-α concentration and DNA fragmentation, as well as the increase in TNF-α plasma levels, occur over the ensuing hours in the striatum and liver of control stroke rats. This suggests cerebral and systemic activation of
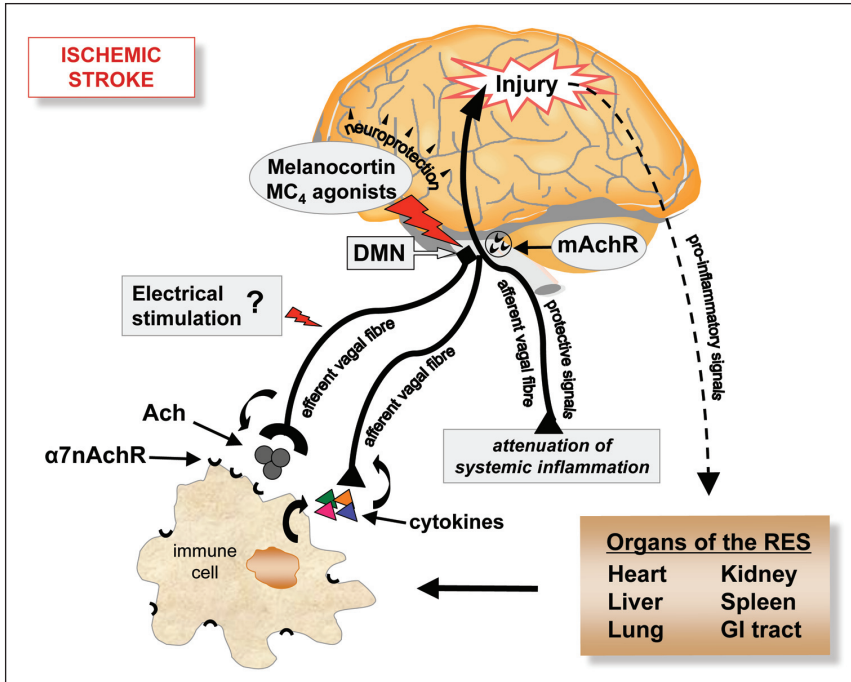
Figure 3. Modulation of the inflammatory response in ischemic stroke, through the cholinergic anti-inflammatory pathway. Low levels of both brain inflammatory molecules spreading outside the blood-brain barrier and inflammatory molecules produced in damaged peripheral tissues, activate afferent signals through ascending vagus nerve fibers (sensory arm of the inflammatory reflex). Brain melanocortin MC_4 receptor agonists, besides to produce strong neuroprotection in the injured brain area, activate the cholinergic anti-inflammatory pathway (motor arm of the inflammatory reflex, that is impaired after stroke), likely by acting on MC_4 receptors in the vagus dorsal motor nucleus (DMN), leading to acetylcholine (Ach) release in organs of the reticuloendothelial system (RES). Acetylcholine released from efferent vagal terminals interacts with α7 subunit-containing nicotinic acetylcholine receptors (α7nAchR) on tissue macrophages and other immune cells surrounding the cholinergic terminals and inhibits the synthesis/release of inflammatory cytokines, with a consequent reduction in cytokine plasma levels and the attenuation of the systemic inflammatory response. Protective signals towards the CNS via the afferent vagal fibers (owing to the activation of the vagus nerve-mediated cholinergic anti-inflammatory pathway) also seem to occur in stroke. It is unknown whether acetylcholine interaction with peripheral muscarinic receptors (mAchR) plays a role in ischemic stroke. Likely, brain muscarinic receptors are also involved in triggering the cholinergic anti-inflammatory pathway. Electrical stimulation has not been assessed in stroke models.

excitotoxic, inflammatory and apoptotic responses. Intraperitoneal treatment with nanomolar doses of NDP-α-MSH 3 to 9 hours after stroke suppresses the excitotoxic, inflammatory and apoptotic cascades at central and peripheral level. Bilateral vagotomy and pharmacological blockade of peripheral nicotinic acetylcholine receptors blunt the neuroprotective effect of NDP-α-MSH.[49] Focal brain ischemia causes, therefore, detrimental effects not only in the brain, but also in the liver. This leads to hypothesize that a protective, melanocortin-activated, vagal cholinergic pathway is likely operative in conditions of ischemic stroke, to modulate cerebral and systemic pathological reactions (Fig. 3). Decreased expression and impaired function of brain muscarinic acetylcholine receptors have been associated with neuron degeneration after

ischemic stroke.[143] This suggests that brain muscarinic receptors may be involved in triggering the cholinergic anti-inflammatory pathway much as in circulatoy shock and myocardial ischemia.

However, the vagus nerve-mediated cholinergic anti-inflammatory pathway is an efferent pathway and its activation by melanocortins can only account for the peripheral protective effects observed by Ottani and coworkers.[49] How can we explain why vagotomy and peripheral nicotinic receptor blockade blunt the protective effect of melanocortins against brain damage? It has been reported that lipopolysaccharide-induced systemic inflammation in conjunction with global cerebral ischemia exacerbates brain damage in rats.[144] This suggests that inhibition of systemic responses could result in cerebral protective effects. Moreover, vagal afferents are widely distributed throughout the CNS and vagus nerve stimulation causes synaptic activation at multiple sites in both cerebral hemispheres.[55] Therefore, protective signals towards the CNS owing to the activation of the vagus nerve-mediated cholinergic anti-inflammatory pathway can be hypothesized in stroke (Fig. 3).

Evidence suggests that in conditions of brain ischemia endogenous melanocortins could exert a role in neuroprotection. A) Melanocortin-treated stroke animals learn more rapidly than sham ischemic ones,[45,46] but after the blockade of melanocortin MC_4 receptors there is a worsening in memory recovery, as compared with ischemic control animals.[45,46] B) Melanocortins increase the anti-inflammatory cytokine IL-10 that modulates the inflammatory cascade.[11,21] Interestingly, it has been reported that low plasma concentrations of IL-10 are associated with early worsening of neurological symptoms in stroke patients.[145] C) α-MSH concentrations in plasma are reduced in patients with acute traumatic brain injury. Consistent with a physiological neuroprotective role of melanocortins, patients with the lowest circulating levels have an unfavourable outcome.[11]

Taken together, these observations point to a neuroprotective role of a cholinergic anti-inflammatory pathway, likely activated by melanocortins, that could be physiologically operative in conditions of ischemic stroke and brain injury, to protect against local and systemic damage. This further supports the important role of the cholinergic anti-inflammatory pathway in the defense mechanisms.

Conclusion

MC_3 and MC_4 receptors are the most abundant MC receptor subtypes within the CNS, being brain distribution of MC_4 receptors broader than that of MC_3.[5,7,11,21] MC_3 and MC_4 receptors also occur in the vagus dorsal motor nucleus and ventral division of the nucleus ambiguous[19,146] and these receptors are believed to play an important role in central regulation of certain body functions.[5,147] This supports the hypothesis that central melanocortins activate efferent vagal fiber-mediated cholinergic anti-inflammatory pathway(s) to protect against damage caused by circulatory shock, myocardial ischemia and ischemic stroke and perhaps following other severe inflammatory insults. Such efferent cholinergic pathways could be specific for different pathological conditions; indeed, in circulatory shock and ischemic stroke this pathway seems to be mediated by peripheral nicotinic acetylcholine receptors, whereas in myocardial ischemia peripheral muscarinic acetylcholine receptors appear to be predominantly involved. However, because MC receptors, including MC_3 and MC_4, are also expressed in numerous peripheral tissues,[7,11,15,21] additional protective influences of melanocortins likely occur via interactions with peripheral melanocortin receptors.

The CNS and peripheral organs communicate via neuronal and humoral pathways.[52,53] After brain injury, immunodepression and inflammation in peripheral organs can occur. Indeed, several cerebral injury types have been shown to be associated with intestinal, pulmonary and hepatic inflammation.[5] The abundant distribution of MC_3 and MC_4 receptors within the CNS, together with the preclinically demonstrated ability of melanocortins acting at central MC_3 and MC_4 receptors to prevent central and peripheral detrimental consequences of a brain inflammatory injury due to ischemia and reperfusion, suggest promising perspectives for the clinical use of melanocortins. These brain receptors could be pharmacological targets for the treatment of several central and peripheral disorders, likely through the activation of the efferent vagal anti-inflammatory pathway(s).[31,40,49]

Several instances of ischemia/reperfusion injuries could take advantage of melanocortin-induced activation of the cholinergic anti-inflammatory pathway, also because melanocortins increase the production and release of the potent anti-inflammatory cytokine IL-10 from monocytes through a β_2-adrenergic receptor-dependent mechanism.[11,52] Indeed, it is well established that melanocortins also stimulate adrenergic transmission and therefore they can centrally modulate local and systemic inflammation through both adrenergic and cholinergic pathways.[5,11] Recent data suggest that the splenic nerve, through a complex mechanism involving adrenergic and cholinergic signals, is required for the cholinergic anti-inflammatory pathway activity against systemic inflammation and the spleen could be a primary source of TNF-α.[70,148]

Investigations designed at determining the molecular mechanisms of the cholinergic anti-inflammatory pathway activation could provide further insight into the neural regulation of inflammation and could help design of superselective activators of such a pathway: agonists at individual brain MC receptors could be significant candidates.

Acknowledgements

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