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In Vitro and In Vivo Induction of Heme Oxygenase 1 in Mouse Macrophages following Melanocortin Receptor Activation

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RAW264.7 cell incubation with adrenocorticotropic hormone (ACTH) led to a time-dependent (4–24 h) and concentration-related (1–100 ng/ml) induction of heme oxygenase (HO-1), and this was a specific effect, because the pattern of expression of other cellular proteins (HO-2, heat shock proteins 70 and 90) was not modified by ACTH. Combined RT-PCR and Western blot analyses revealed expression of the melanocortin receptor (MC-R) types 1 and 3, but not 4, in these cells. However, use of more selective agonists (including melanotan (MTII)) indicated a predominant role for MC3-R in the induction of HO-1 expression and activity. Relevantly, ACTH and MTII induction with primary peritoneal macrophages (Mφ) also induced HO-1 expression. The potential link between MC3-R dependent cAMP formation and HO-1 induction was ascertained by the following: 1) ACTH and MTII produced a concentration-dependent accumulation of cAMP in RAW264.7 cells, and 2) whereas a selective inhibitor of cAMP-dependent protein kinase A abrogated ACTH- and MTII-induced HO-1 expression, a soluble cAMP derivative promoted HO-1 induction both in RAW264.7 cells and primary Mφ. HO-1 induction in peritoneal Mφ was also detected following in vivo administration of MTII, and appeared to be functionally related to the antimigratory effect of this melanocortin, as determined with a specific inhibitor (zinc protoporphyrin IX). In conclusion, this study highlights a biochemical link between MC-R activation and HO-1 induction in the Mφ, and proposes that this may be of functional relevance in determining MC-R-dependent control of the host inflammatory response. The Journal of Immunology, 2005, 174: 2297–2304.

Melanocortin peptides (e.g., α-melanocortin-stimulating hormone (α-MSH)1) have long been reported to possess anti-inflammatory effects in many experimental models of acute (1, 2) and chronic inflammation, including inflammatory bowel disease (3), allergy (4), joint arthritis (5, 6), and systemic inflammation (endotoxemia) (7). Interestingly, recent trials with α-MSH have confirmed the positive indication for this compound in controlling human disease (8), thereby reinforcing the potential impact of this line of research. Because α-MSH represents the first 13 aa within the adrenocorticotropic hormone (ACTH) sequence (39 aa in total) (9), it is important to recall the efficacy of the longer polypeptide in controlling rheumatoid arthritis (10).

At the molecular level, the effects of these anti-inflammatory hormones and synthetic derivatives on target cells are brought about by activation of a subgroup of G protein-coupled receptor, termed melanocortin receptors (MC-R). Five MC-Rs have been identified so far, and all of these receptors are positively coupled to adenylyl cyclase such that their activation leads to increases in intracellular cAMP (9, 11). Following this early event of cAMP formation, and also perhaps partly independently from it, melanocortin peptides have been shown to down-regulate NF-κB activation and consequent cytokine synthesis (12, 13). The C terminus sequence (i.e., aa 11–13) of α-MSH outside the common core and modifications of it (14, 15) have been shown to block cytokine functions (1), rather than synthesis and release (16).

Heme oxygenase (HO-1) is the rate-limiting enzyme in heme catabolism with consequent generation of biliverdin (then converted to bilirubin), free iron, and carbon monoxide. Three mammalian HO isoforms have been identified, one of which, HO-1, is a stress responsive protein endowed with important cytoprotective effects (for a recent review, see Ref. 17). In addition, macrophage (Mφ) HO-1 expression is part of the repairing processes that occur during resolving inflammation leading to healing and tissue repair (18). It is possible that at least some of the cytoprotective and anti-inflammatory actions of HO-1 are due to the controlled local liberation of carbon monoxide, able to signal through the cyclic GMP pathway (17) and inhibit cytokine synthesis (19). In addition, the other catabolite bilirubin is also endowed with antioxidant and anti-inflammatory effects, for instance, its application inhibits LPS-induced selectin expression in the vasculature, thus affecting leukocyte recruitment (20).

The present study was undertaken to assess a potential functional link between MC-R-dependent cAMP formation and HO-1 induction in Mφ. Most of the experiments have been conducted with the RAW264.7 Mφ cell line, both for data consistency and ease of manipulation (and reduction in animal sacrifice); however, crucial experiments have been repeated with primary Mφ. Importantly, in vivo experimentation not only confirmed the biochemical link between MC-R activation and HO-1 induction, but also provided a functional relevance to this interaction. We conclude that MC3-R activation, and possibly activation of other MC-R subtype.
as well, can bring about anti-inflammatory effects mediated, at least in part, by HO-1 induction.

Materials and Methods

In vitro experimental section

Cell culture and treatments. RAW264.7 cells (ECACC, Sigma-Aldrich) or primary Mφ were cultured in DMEM or RPMI 1640, respectively, supplemented with 10% heat-inactivated FBS in a humidified atmosphere supplied with 5% CO2 and 95% air, until 80% confluence was attained for use. Before experiment, FBS in culture was reduced to 1% for 24 h, and subsequently all experiments were performed under these conditions. Cells were treated with (positive control), ACTH (3–300 ng/ml), or MTI (0.3–300 μg/ml) in 1 mM isobutylmethylxanthine in serum-free medium and incubated for 30 min at 37°C in humidified atmosphere of 5% CO2 and 95% air. A nontreated group with serum-free medium alone was also set up under the same incubation condition as negative control. Cell supernatants were then removed and cells were lysed. Intracellular cAMP elevation was measured with a commercially available enzyme immunoassay using a standard curve constructed with 0–3200 fmol/ml cAMP (Amersham Biosciences).

HO-1 activity assay. RAW264.7 cells (2.5 × 10^6 cells) were treated with PBS, LPS (1 μg/ml; positive control), ACTH (100 ng/ml), or MTI (10 μg/ml) for 8 h. Cell pellets were resuspended in magnesium supplemented peritoneal Mφ (5 × 10^6 cells) were isolated in accordance to the protocol in the RNeasy Mini kit (Qiagen). In brief, cells were lysed in a guanidine isothiocyanate- and 2-ME-containing RLT buffer and passed through a 20-gauge needle five times to disrupt the Mφ. DNase was then applied onto membrane and incubated for 15 min to digest any remaining DNA contaminants in the samples. The membrane was subsequently washed with RW1 and RPE buffers supplied in the kit at 8000 × g to remove contaminating DNA and membrane. The total RNA yield and purity were assessed spectrophotometrically at 260 and 280 nm. Aliquots of RNA (1 μg) were used in the RT-PCR preparation by denaturation at 65°C for 5 min in presence of 1 μg of random primers. The RT-PCR preparations were subsequently generated into cDNA by incubation with 5′ AMV-RT buffer (3 μl), RNAsin enzyme, and AMV-RT enzymes at 42°C for 20 min. RT-PCR amplifications were performed in 25 μl aliquots of cDNA (1 μg) with 1 μl of each of the primers, 1.5 μM MgCl2, Taq polymerase, classic 18 s ribosomal RNA primers (as internal control; giving a product of 324 bp), and MC-R primers (0.2 μM final concentration). The murine MC-R primer sequences were as follows: MC1-R, 5′-GTC CAG TCT CGT CTT CGG-3′ and 5′-TCT TCA GGA GCC TGT GGT CT-3′ (forward and reverse) with fragment amplification of 825 bp in length; MC3-R, 5′-GCC TGT CTT CGT TTC CG-3′ and 5′-GCC GTG TAG CAG ATG CAG TA-3′ (forward and reverse) with fragment amplification of 820 bp in length; MC4-R, 5′-ATC CAT TTG CAG CTG TT-3′ and 5′-ATG AGA CAT GAA CAG CAC GGC C-3′ (forward and reverse) with fragment amplification of 445 bp in length (21). Primers were purchased from Applied Biosystems. Classic 18 s ribosomal RNA primers and random primers were purchased from Promega. The total volume of each reaction was made up to 50 μl with RNA-free distilled water. The cycle parameters were as follows: initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation (94°C for 45 s, annealing (60°C for 30 s), extension (72°C for 2 min)), and a final extension of 72°C for 10 min. Amplified products were visualized by ethidium bromide fluorescence in 1% agarose gels.

In vivo experimental section

Male Swiss Albino mice (body weight, 20–22 g) were purchased from Bantin & Kingman. Mice were maintained on a standard chow pellet diet with tap water ad libitum using a 12-h light/dark cycle. Animal experimental work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

For the in vivo experiments, peritoneal cells (>80% Mφ) were collected from culled mice by injection of 3 ml of PBS supplemented with 1 mM EDTA and gentle massage of the peritoneal cavity. On average, 2.5 × 10^6 cells and 1.5 × 10^6 Mφ were recovered from each peritoneal cavity (Ref. 22). In vivo, mice were treated with an anti-inflammatory dose of MTI (10 μg; Ref. 22), and peritoneal cells were harvested at different times postadministration. For the inflammation model, we selected the urate crystal induction model in accordance to the protocol in the RNeasy Mini kit (Qiagen). In brief, cells were lysed in a guanidine isothiocyanate- and 2-ME-containing RLT buffer and passed through a 20-gauge needle five times to disrupt the Mφ. DNase was then applied onto membrane and incubated for 15 min to digest any remaining DNA contaminants in the samples. The membrane was subsequently washed with RW1 and RPE buffers supplied in the kit at 8000 × g to remove contaminating DNA and membrane. The total RNA yield and purity were assessed spectrophotometrically at 260 and 280 nm. Aliquots of RNA (1 μg) were used in the RT-PCR preparation by denaturation at 65°C for 5 min in presence of 1 μg of random primers. The RT-PCR preparations were subsequently generated into cDNA by incubation with 5′ AMV-RT buffer (3 μl), RNAsin enzyme, and AMV-RT enzymes at 42°C for 20 min. RT-PCR amplifications were performed in 25 μl aliquots of cDNA (1 μg) with 1 μl of each of the primers, 1 μM MgCl2, Taq polymerase, classic 18 s ribosomal RNA primers (as internal control; giving a product of 324 bp), and MC-R primers (0.2 μMfinal concentration). The murine MC-R primer sequences were as follows: MC1-R, 5′-GTC CAG TCT CGT CTT CGG-3′ and 5′-TCT TCA GGA GCC TGT GGT CT-3′ (forward and reverse) with fragment amplification of 825 bp in length; MC3-R, 5′-GCC TGT CTT CGT TTC CG-3′ and 5′-GCC GTG TAG CAG ATG CAG TA-3′ (forward and reverse) with fragment amplification of 820 bp in length; MC4-R, 5′-ATC CAT TTG CAG CTG TT-3′ and 5′-ATG AGA CAT GAA CAG CAC GGC C-3′ (forward and reverse) with fragment amplification of 445 bp in length (21). Primers were purchased from Applied Biosystems. Classic 18 s ribosomal RNA primers and random primers were purchased from Promega. The total volume of each reaction was made up to 50 μl with RNA-free distilled water. The cycle parameters were as follows: initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation (94°C for 45 s, annealing (60°C for 30 s), extension (72°C for 2 min)), and a final extension of 72°C for 10 min. Amplified products were visualized by ethidium bromide fluorescence in 1% agarose gels.
peritonitis (23), extensively used to investigate the anti-inflammatory actions of melanocortins (e.g., see Ref. 21). In this case, at time 0, mice received 3 mg of monosodium urate crystals alone or together with MTII. Eight hours later, some animals were treated with the HO-1 inhibitor zinc protoporphyrin IX (ZnPPIX) given at the dose of 10/mol/kg i.p (20, 24).

Peritoneal cavities were washed at the 24-h time point post-crystal injection, and the number of PMN migrated was determined following staining in Turk’s and counting in a Neubauer hematocytometer as described (23).

Statistical analysis

All values are expressed as mean ± SEM, with an n number of mice for the in vivo experiments. For in vitro analysis, experiments were repeated at least with three distinct preparations (i.e., RT-PCR and Western blotting analyses). Statistical analysis was assessed either by Mann-Whitney U test (for blot analyses) or one-way ANOVA followed by Bonferroni test (for the in vivo experimentation). In all cases, a value of p < 0.05 was considered significant to reject the null hypothesis.

Results

RAW264.7 cells express functional MC3-R

At the beginning of the study, we confirmed that RAW264.7 cells could act as a reliable surrogate for primary Mφs. In line with that reported for mouse peritoneal Mφs (21), RAW264.7 cells expressed MC3-R mRNA and protein (Fig. 1). Immunocytochemistry confirmed receptor expression on the cell surface of RAW264.7 cells and Mφs, apparently to the same extent (Fig. 1c). RT-PCR was used to monitor the expression of other receptors of the family, concluding that RAW264.7 cells expressed MC1-R, but not MC4-R, mRNA (Fig. 1b).
Addition of the mixed MC3/4-R agonist MTII to RAW264.7 cells provoked a concentration-dependent accumulation of intracellular cAMP (Fig. 2). The synthetic derivative was at least two orders of magnitude less potent than the natural agonist ACTH. Altogether, these signaling profiles are in line with those derived from experimentation with primary Mφ (21, 22).

**MC3-R and HO-1 induction**

Induction of cAMP formation has long been recognized as the major signal conveyed by MC-R activation (9), and because this signaling molecule has been linked to HO-1 induction in rat hepatocytes (25), we asked whether MC3-R could also modulate expression of this anti-inflammatory enzyme. To address this, RAW264.7 cells were incubated with different melanocortin peptides or LPS, as positive control, for longer time points. Fig. 3 reports the data obtained at 8 h, in which ACTH or α-MSH or the synthetic derivatives ACTH4–10 or MTII, caused selective induction of HO-1. In contrast, activation of MC3-R by the MTII agonist did not alter the basal expression of HO-2, HSP-70 and -90, COX-2, and iNOS (Fig. 3). Cells incubated with LPS displayed higher HO-1, iNOS, and COX-2, but not HO-2 and HSP-70/90, protein expression (Fig. 3). Cumulative densitometric analyses of these experiments are in Table 1.

HO-1 induction by MTII was time dependent, with a plateau at 8 h (Fig. 4). Similar findings were obtained with ACTH and RAW264.7 cells, as well as with primary Mφ in culture (not shown for either experiment). In addition, MTII-induced HO-1 protein expression was associated with augmented enzymatic activity, particularly evident 24 h post-MTII incubation. For instance, the HO-1 activity (picomoles of bilirubin/milligram of protein/hour) was 523 ± 81 in control cells and raised to 764 ± 88 and 845 ± 45 for RAW264.7 cells treated for 24 h with 100 ng/ml ACTH or 10 μg/ml MTII, respectively (n = 4; p < 0.05) with intermediate values of enzymatic activity being measured at 8 h (data not shown).

Finally, the functional link between early postreceptor activation signaling responses and more downstream and delayed events, such as HO-1 induction, was established with specific inhibitors and mimicking molecules. For instance, the PKA inhibitor H-89 abrogated ACTH- or MTII-mediated HO-1 induction in RAW264.7 cells and primary Mφ (Fig. 5). Similar results were obtained with another PKA inhibitor, the structurally unrelated permeable peptide 14–22 amide (data not shown). Conversely, cell incubation with the stable cAMP analog dibutyryl-cAMP caused HO-1 protein up-regulation, as detected at 8 h, in a PKA-dependent fashion (Fig. 5).

**In vivo relevance of MC3-R mediated HO-1 induction**

Once we assessed that the cAMP-dependent HO-1 induction in RAW264.7 cells occurred also in primary cultures of mouse peritoneal Mφ, we next sought to provide an in vivo relevance to these findings. Intraperitoneal administration of an anti-inflammatory dose of MTII (10 μg per mouse (22, 26)) provoked a time-dependent induction of HO-1 in resident Mφ, as detected ex vivo by Western blotting analysis (Fig. 6a). Cumulative data for multiple animals as reported in Fig. 6b show a peak of induction at 8 h postinjection, reminiscent of the data obtained in vitro with RAW264.7 cells (Fig. 4) and primary Mφ (data not shown).

The functional relevance of the MC3-R/HO-1 axis was tested by means of the HO-1 inhibitor ZnPPIX. The inflammatory response to urate crystal at 24 h (23) was used here, injecting mice with MTII at time 0 and with the HO-1 inhibitor at 8 h. Fig. 7 reports

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**Table I. Cumulative densitometric analysis of protein expression in RAW264.7 cells incubated with melanocortin agonists**

<table>
<thead>
<tr>
<th>Protein</th>
<th>PBS</th>
<th>ACTH</th>
<th>α-MSH</th>
<th>ACTH4–10</th>
<th>MTII</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>88 ± 15*</td>
</tr>
<tr>
<td>HSP-90</td>
<td>52 ± 11</td>
<td>63 ± 17</td>
<td>57 ± 11</td>
<td>42 ± 11</td>
<td>51 ± 8</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>COX-2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>81 ± 18*</td>
</tr>
<tr>
<td>HSP-70</td>
<td>41 ± 3</td>
<td>43 ± 11</td>
<td>36 ± 8</td>
<td>35 ± 10</td>
<td>43 ± 4</td>
<td>40 ± 15</td>
</tr>
<tr>
<td>HO-2</td>
<td>80 ± 12</td>
<td>88 ± 4</td>
<td>86 ± 9</td>
<td>78 ± 11</td>
<td>85 ± 9</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>HO-1</td>
<td>6 ± 3</td>
<td>48 ± 15*</td>
<td>47 ± 11*</td>
<td>46 ± 5*</td>
<td>63 ± 14*</td>
<td>82 ± 22*</td>
</tr>
</tbody>
</table>

*RAW264.7 cells were incubated for 8 h with PBS, ACTH (100 ng/ml), α-MSH (10 μg/ml), ACTH4–10 (100 μg/ml), MTII (10 μg/ml), or LPS (1 μg/ml; positive control) prior to assessment of protein expression with specific Abs by Western blotting. Data are mean ± SEM of three distinct experiments, and express arbitrary units from densitometric analysis. n.d., Not detectable.

* p < 0.05 vs PBS-treated cells.
As expected, MTII exerted an antimigratory effect on PMN influx as measured 24 h postcrystal injection (50% inhibition; \(p < 0.05\)). The anti-inflammatory nature of HO-1 in this model of peritonitis was confirmed by the augmented PMN influx (70% increase; \(p < 0.05\)) measured in mice treated with ZnPPIX (Fig. 7). Importantly, when ZnPPIX was given to mice previously treated with MTII, the antimigratory effect of the melanocortin derivative was no longer evident. These in vivo data indicate a major functional role for MTII-induced HO-1 at least with respect to the delayed anti-inflammatory effect measured 24 h post-crystal inflammation.

**Discussion**

This study demonstrates a previously unknown link between Mφ MC3-R activation and induction of the anti-inflammatory enzyme HO-1. Activation of this receptor by MTII, and the less selective agonist ACTH, produces transient alterations in intracellular cAMP that are temporally related to HO-1 up-regulation in a PKA-dependent fashion. In vivo, the MC3-R/HO-1 connection is functionally operative in bringing about the antimigratory effect of a melanocortin peptide.

Since the initial studies with \(-\)MSH (27), melanocortin peptides have been shown to represent an important component of the counterregulatory systems that operate in the host to dampen and control the inflammatory reaction. Several studies conducted in experimental animals with models of acute and subacute inflammation (1, 28), in some cases supported by human data (29), have shown how \(-\)MSH and other melanocortin peptides are endowed with potent inhibitory and anti-inflammatory properties (8). This field attracted much more interest once specific receptors were cloned and shown to mediate the actions of several melanocortins, including the naturally occurring ACTH and \(-\)MSH, on different target cells. MC-R belongs to the family of G protein-coupled receptors, and their activation leads to adenylate cyclase-mediated conversion of ATP into cAMP (9, 11). Thus, accumulation of cAMP in target cells buffers cell activation with a marked effect on the production of proinflammatory cytokines (12, 13, 21). Therefore, targeting specific MC-R could certainly represent a novel...
Inhibitory properties, at least at the level of the M tors, allowed us to pinpoint MC3-R as a major determinant for the release of an array of cytokines and chemokines (21, 22). Comparing the effects produced by agonists and antagonists, to the inhibition of LPS-induced selectin expression in the vasculature, cytokine synthesis (19), whereas the product of the enzymatic reaction, bilirubin, is endowed with antioxidant and anti-inflammatory effects, causing, for instance, inhibition of LPS-induced selectin expression in the vasculature, thus affecting leukocyte recruitment (20).

In all cases, most of the anti-inflammatory properties of HO-1 take place in the Mφ (18, 38). Thus, in the final part of the study, we sought to provide a functional role for MTII-induced HO-1 in an experimental model of inflammation. Our choice fell on the urate crystal peritonitis because it is characterized by a prolonged PMN influx (23) and has been shown to be sensitive to melatonin peptide administration (21, 22). Initially, we tested whether MTII could up-regulate Mφ HO-1 following i.p. administration: this agonist was chosen because of its selectivity, at least partial, toward MC3-R (31), long half-life (39), and efficacy in this model of peritonitis (22, 26). The profile of induction obtained was similar to that obtained following in vitro incubation with this cell type, with a peak of HO-1 expression at the 8-h time point. Next, the effect of the HO-1 inhibitor ZnPPIX on MTII-mediated inhibition of urate crystal-induced PMN recruitment into the mouse peritoneal cavity was tested, by giving the inhibitor 8 h into the experiment on rat hepatocytes (25), MTII-mediated HO-1 induction was genuinely due to cAMP/PKA signaling, a fact that was confirmed also with primary Mφ cultures. It is of interest that, during the preparation of this paper, a study showing ACTH induction of HO-1 in a mouse adrenocortical cell line was published (33). It therefore seems that activation of more than one MC-R (certainly MC3-R and MC2-R, the latter being selectively expressed in adrenal cells, and possibly all the other receptors) in target cells can lead to HO-1 up-regulation, thereby making this biochemical link a more general phenomenon. Subsequently, we investigated the potential functional consequences of this induction in experimental inflammatory settings.

Mφ HO-1 has long been recognized to possess cytoprotective properties, and in the context of inflammation, this enzyme catalyzes heme. In fact, it has an important scavenger function during the resolution and healing phases of the acute inflammatory reaction, because HO-1 disposes excess heme of enzymatic source or from the hemoglobin lost by dying erythrocytes extravasated as part of the exudation process (17). In an acute resolving inflammatory response, that is, rat pleurisy, HO-1 is selectively expressed in Mφ, and not PMN, during the late proresolving phase of the response (18). Interestingly, the model we used here is also acute and naturally resolves (23). Recent years have seen an appreciation of more subtle and homeostatic functions of Mφ HO-1 together with specific networks among anti-inflammatory mediators. For instance, IL-10 acts via HO-1 to protect from excessive Mφ activation as seen in experimental endotoxiaemia (34), and the scavenger receptor CD163 controls both IL-10 and HO-1 synthesis and expression in human Mφ (35). In addition, Mφ HO-1-derived CO can produce several biological actions, mostly protective at the concentration levels found in exudates (17, 36). The cytoprotection afforded by novel CO-releasing molecules (37) emphasizes the pivotal role that CO produced by HO-1 from heme degradation may be playing in the pathophysiological context of a resolving inflammatory process. From a mechanistic point of view, HO-1-derived carbon monoxide inhibits cytokine synthesis (19), whereas the product of the enzymatic reaction, bilirubin, is endowed with antioxidant and anti-inflammatory effects, causing, for instance, inhibition of LPS-induced selectin expression in the vasculature, thus affecting leukocyte recruitment (20).
In conclusion, this study indicates that HO-1 induction in MØ might be a major arm in the complex series of effects that are produced by melanocortin peptides acting at their MC-R. Our analyses on MØ function, in the present and previous studies, suggest that MC3-R is the major receptor determinant for transducing the anti-inflammatory actions of these peptides on MØ, although it is clear that we could also detect MC1-R in RAW264.7 cells, in analogy to Star et al. (32). However, irrespective of the specific MC-R, a more general picture is emerging in which MC-R activation on the MØ cell surface leads to cAMP formation and PKA activation. This signaling pathway produces at least two downstream events: inhibition of cytokine synthesis and release from stimulated MØ (in the presence of an inflammogen), which is evident within the first 2 h, and then up-regulation of HO-1 from 4 to 6 h post-MC-R activation. Importantly, the latter effect is achieved by the melanocortin peptide itself (i.e., in the absence of an inflammogen or MØ activator), suggesting that MC-R activation favors the acquisition of the anti-inflammatory proresolving phenotype by the MØ (a phenomenon originally described for glucocorticoids (40)). Fig. 8 schematizes this model of two hits, or anti-inflammatory mechanisms, activated by MC3-R agonists.

Figure 8. Schematic representation of the anti-inflammatory effects switched on in MØ by MC3-R activation. Resident MØ express MC3-R, and agonism at this receptor leads to accumulation of cAMP with consequent activation of PKA. This results in at least two major effects: the paninhibition exerted by these peptides on stimulated cytokine synthesis and release, evident already after short incubation times (<4 h), and the induction of the stress protein HO-1, which requires longer incubation times (>4–6 h, optimal at 8 h as reported in this study). Either mechanism of action can impact on the host inflammatory response and be responsible for the potent and reproducible antiinflammatory effects that melanocortin peptides exert in the context of acute local and systemic inflammation. See end of Discussion for more details.

More and more evidence indicates the existence of networks or interconnections among several anti-inflammatory pathways. We propose that the biochemical and functional link reported in the present study for MC3-R activation and consequent HO-1 expression might be of help in the development of melanocortin peptides or derived small chemical entities endowed with potent and prolonged anti-inflammatory effects.

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