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Structure-activity analysis for the effects of γ-MSH/ACTH-like peptides on cerebral hemodynamics in rats

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Abstract

In a previous structure-activity analysis we have shown that the γ-melanocyte-stimulating hormones (γ-MSHs) and structurally related adrenocorticotropic hormone (ACTH) fragments share an amino-acid sequence which is determinant for the effects of these peptides on peripheral hemodynamics, viz. a pressor and a tachycardiac response, in conscious rats. We now investigated whether these structural features are also important for the effects of these peptides on cerebral hemodynamics in urethane-anesthetized rats. After intracarotid and intravenous administration, the 'mother' peptides, Lys-γ_2-MSH and γ_2-MSH, and, with a 10-fold lower potency, ACTH-(4–10), caused a dose-dependent pressor and tachycardiac response, as well as an increase in extra- and intracranial blood flow and microcirculatory cerebrocortical blood flow. Removal of C-terminal amino acids resulted in γ-MSH-fragments which were devoid of effects on peripheral and central hemodynamics. Fragments of γ_2-MSH which were shortened at the N-terminal side (γ-MSH-(4–12) and γ-MSH-(5–12)) were less potent than γ_2-MSH, but had an intrinsic activity similar to that of γ_2-MSH with respect to the pressor and tachycardiac effect. However, the potency and intrinsic activity of these shortened fragments on intracerebral hemodynamic parameters were the same as those of γ_2-MSH. This suggests that different mechanisms (e.g., site of action and/or melanocortin receptor subtype) are involved in the cerebral hemodynamic effects of the melanocortins and in their peripheral hemodynamic effects. Surprisingly, removal of an additional residue, His^5, resulting in the fragment γ-MSH-(6–12), led to full restoration of potency with respect to extracranial blood flow, blood pressure and heart rate. Neither the structurally related analog, [Nle^4,D-Phe^7]α-MSH (NDP-MSH), nor ACTH-(1–24) was able to induce a pressor effect or cerebral hemodynamic effects. In contrast, both compounds had a depressor effect. It is concluded that the C-terminal amino acids in the structure of γ-MSH/ACTH-like peptides are essential for efficacy for the central hemodynamic effects, i.e., the increase in intracerebral (microcirculatory) blood flow. However, in contrast to what holds for the peripheral hemodynamic features, the N-terminal sequence has hardly any influence on potency or efficacy. The results with NDP-MSH and ACTH-(1–24) and the other fragments lead us to postulate that it is not one of the five known subtypes of melanocortin receptors which mediates the hemodynamic effects of the melanocortins, but an additional, still unidentified subtype. A clue for the elucidation of such a receptor might be found in the structural features of γ-MSH-(6–12) that appear to be very important determinants for the effectiveness to alter peripheral and central hemodynamics.

Keywords: γ-MSH (γ-melanocyte-stimulating hormone) fragment; ACTH (adrenocorticotropic hormone) fragment; Blood pressure; Heart rate; Cerebral blood flow; Structure-activity relationship; (Rat)

1. Introduction

Pro-opiomelanocortin (POMC)-derived melanotropins and corticotropins, after intravenous (i.v.) administration to conscious rats, can affect blood pressure and heart rate. Structure-activity analysis using a series of (partially) homologous peptides (see Table 1) showed that removal of C-terminal amino acids from γ_2-melanocyte-stimulating hormone (γ_2-MSH), one of the most potent pressor and cardioaccelerator peptides of the class of melanotropins (Gruber et al., 1984, 1985; Klein et al., 1985; Gruber and Callahan, 1989; Sun et al., 1992; De Wildt et al., 1993), yields γ-MSH-fragments devoid of activities on blood pressure and heart rate (Van Bergen et al., 1995). Shortening at the N-terminal side of γ_2-MSH yields fragments

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Table 1
Amino-acid sequences of various γ-MSH/ACTH-fragments

Lys-γ_2-MSH	Lys-	Tyr^1-	Val^2-	Met^3-	Gly^4-	**His^5-**	**Phe^6-**	**Arg^7-**	**Trp^8-**	Asp^9-	Arg^{10}-	Phe^{11}-Gly^{12}
γ_2-MSH		Tyr^1-	Val^2-	Met^3-	Gly^4-	**His^5-**	**Phe^6-**	**Arg^7-**	**Trp^8-**	Asp^9-	Arg^{10}-	Phe^{11}-Gly^{12}
γ-MSH-(4-12)					Gly^4-	**His^5-**	**Phe^6-**	**Arg^7-**	**Trp^8-**	Asp^9-	Arg^{10}-	Phe^{11}-Gly^{12}
γ-MSH-(5-12)						**His^5-**	**Phe^6-**	**Arg^7-**	**Trp^8-**	Asp^9-	Arg^{10}-	Phe^{11}- Gly^{12}
γ-MSH-(6-12)							**Phe^6-**	**Arg^7-**	**Trp^8-**	Asp^9-	Arg^{10}-	Phe^{11}- Gly^{12}
γ-MSH-(3-8)				Met^3-	Gly^4-	**His^5-**	**Phe^6-**	**Arg^7-**	**Trp^8**			
ACTH-(4-10)				Met^4-	Glu^5-	**His^6-**	**Phe^7-**	**Arg^8-**	**Trp^9-**	Gly^{10}		
ACTH-(4-9)				Met^4-	Glu^5-	**His^6-**	**Phe^7-**	**Arg^8-**	**Trp^9**			
NDP-MSH	Ac-	Ser^1-	Tyr^2-Ser^3-	Nle^4-	Glu^5-	**His^6-**	**D-Phe^7-**	**Arg^8-**	**Trp^9-**	Gly^{10}-	Lys^{11}-	Pro^{12}- Val^{13}- NH_2
ACTH-(1-24)		Ser^1-	Tyr^2-Ser^3-	Met^4-	Glu^5-	**His^6-**	**Phe^7-**	**Arg^8-**	**Trp^9-**	Gly^{10}-	Lys^{11}-	Pro^{12}- Val^{13}-- Pro^{24}

The MSH 'core' sequence His-Phe-Arg-Trp is highlighted in bold.

with reduced potency but an intrinsic activity similar to that of γ_2-MSH (Van Bergen et al., 1995). Adrenocorticotropic hormone-(4–10) (ACTH-(4–10)), which is structurally closely related to γ_2-MSH, like the γ-MSHs melanotropins, causes an increase in blood pressure and heart rate with comparable intrinsic activity but lower potency (Klein et al., 1985; Gruber and Callahan, 1989; De Wildt et al., 1993; Van Bergen et al., 1995). As was the case for γ_2-MSH, the C-terminal part of ACTH-(4–10) (the Gly^{10} residue) carries the intrinsic activity of this heptapeptide, and the N-terminal part (the Met^4-Glu^5 dipeptide) carries the potency (Van Bergen et al., 1995). Structurally related melanotropic and corticotropic peptides with a C-terminal extension, such as α-MSH and its stable analog, [Nle^4,D-Phe^7]α-MSH (NDP-MSH) (see Table 1), respectively, lack pressor and cardioaccelerator actions in conscious rats (De Wildt et al., 1993; Van Bergen et al., submitted). Interestingly, i.v. administered ACTH-(1–24) produced a dose-dependent decrease in blood pressure in combination with a reflex tachycardia (Nakamura et al., 1976; Van Bergen et al., submitted).

Recently we have demonstrated that γ_2-MSH causes cerebral hemodynamic effects, among them a marked increase in macro- and microcirculatory cerebral blood flow, in urethane-anesthetized rats after both i.v. and intracarotid arterial (i.car.) administration (De Wildt et al., 1995). This increase in intracerebral blood flow is probably independent of the γ_2-MSH-mediated higher perfusion pressure which is a result of its pressor action (De Wildt et al., 1995). Furthermore, there is evidence that the enhancement of microcirculatory neocortical blood flow by the peptide is (partly) of central origin (De Wildt et al., 1995).

The aim of the present study was to investigate whether the relation between structural features of melanocortins and corticotropins and their effects on cerebral hemodynamics is similar to structure-activity relationships of these peptides for peripheral hemodynamic effects.

2. Materials and methods

2.1. Animals

The experiments were carried out with male Wistar rats (U:WU cpb), weighing between 225–325 g. Prior to surgery the rats were housed three to a cage. Food pellets and tap water were provided ad libitum. Room temperature was kept at 20 ± 1°C with lights on from 7:00 a.m. to 7:00 p.m. and a relative humidity of 50–60%.

The design of the experiment was approved by the Experimental Animal Board of the Medical Faculty of Utrecht University.

2.2. Procedure

The rats were anesthetized with urethane (10% dissolved in 0.9% NaCl solution) at a dose of 1.3 ml/100 g body weight intraperitoneally (Maggi and Meli, 1986). Body temperature was recorded with a rectal probe and maintained at 37°C with a homeothermic blanket system (Harvard Apparatus). Tracheoctomy was performed with polythene tubing (i.d. 1.57 mm, o.d. 2.08 mm) and the rats were artificially ventilated with room air and a small animal respirator (Model 683, Harvard Apparatus) delivering 75 strokes/min at 2.5–3.0 ml/stroke. When necessary, ventilation was adjusted to maintain P_aCO_2 and pH within the range of 33.0–38.0 mmHg and 7.35–7.45, respectively.

For the measurement of blood pressure and heart rate, and to obtain arterial blood samples for blood gas analysis, a femoral artery was cannulated. The cannula consisted of a polythene tube (PP25, i.d. 0.40 mm, o.d. 0.80 mm) melted into a polyethylene tube (PE50, i.d. 0.58 mm, o.d. 0.965 mm). The cannula was filled with heparin solution (50 IU/ml).

For i.v. administration of peptides the ipsilateral femoral

vein was cannulated with PE50. This cannula was filled with saline. For i.car. administration of peptides the external carotid artery was retrogradely cannulated with PP25 tubing melted into PE50 tubing so that the tip of the catheter was positioned at the carotid bifurcation at the origin of the internal carotid artery. Care was taken not to damage the nerves of the carotid body. Except for the third series of experiments, the side branches of the contralateral external carotid artery (vide infra) were either coagulated or ligated in order to prevent blood and peptides from reaching extracerebral areas during i.car. administration of peptides. The cannula was filled with a heparin solution (50 IU/ml) in both experiments.

2.3. Measurement of blood pressure, heart rate, and blood gas variables

Arterial blood pressure and heart rate were measured by connecting the aortic cannula to a pressure transducer (Viggo-Spectramed, disposable DTX/plus). The pressure transducer was connected to a pressure signal pre-amplifier/biotachometer (Instrument Service, Utrecht University) and a data acquisition/processing system (vide infra).

Arterial blood gasses and pH were measured in blood samples (60 μl) with a blood gas analyzer (Ciba Corning Type 288) before i.v. and i.car. administration.

2.4. Measurement of total and internal carotid blood flow

Carotid blood flow was measured continuously with an ultrasonic transit-time flowmeter (T 206 small animal flowmeter, Transonic Systems), a system which provides both a direct measure of volume flow (ml/min), independent of flow profile and vessel diameter, and a real-time recording of either pulsatile flow or calculated mean flow. The common carotid artery is an easily accessible vessel for the acute measurement of carotid blood flow with this flow measuring system (Honda et al., 1988; De Wildt et al., 1995). The total (intra- and extracerebral) carotid blood flow (CF_{tot}) was measured from the ipsilateral common carotid artery. To exclude the extracerebral component of the carotid flow, flow measurements were performed when all side branches of the carotid artery (occipital, pterygopalatine, superior thyroid, and distal external carotid arteries), except the internal carotid artery, were ligated (Okazaki et al., 1992; De Wildt et al., 1995). Therefore, the flow measured in the contralateral, ligated common carotid artery will reflect flow in the internal carotid artery (CF_{int}) (De Wildt et al., 1995). A small perivascular probe (size 1RB or 1RS, Transonic Systems) was placed around the vessels and filled with acoustic gel. The cables of the probes were attached to the sternohyoideus with a suture to secure them in a stable position. The flowmeter was connected to a data acquisition/processing system (vide infra).

2.5. Measurement of cerebral blood flow

Microcirculatory cerebral blood flow was measured continuously by laser-Doppler flowmetry. A Perimed flowmeter (Periflux PF3, Stockholm, Sweden) was used according to the method described by Iadecola and Reis (1990). Laser-Doppler flowmetry allows continuous and non-invasive measurement of the microvascular blood flow in superficial brain regions. Even though this technique does not measure strictly blood flow, but movements of red blood cells in the microcirculation (Edvinsson et al., 1993), the values obtained in the rat cerebral cortex correlate well with those obtained with established methods for measuring cerebral blood flow (Skarphedinsson et al., 1988; Haberl et al., 1989). The flowmeter was equipped with a 2-mW helium-neon laser with a wavelength of 632.8 nm. Flow values are expressed in arbitrary units (perfusion units, PU). The contralateral parietal bone was exposed and a small square (3 × 2 mm) was drilled 1 mm laterally and 1 mm caudally to the bregma. The dura mater was left intact. The square was frequently cooled with saline during drilling to prevent excessive heating. A needle probe (PF 302, tip diameter 0.45 mm) was placed in a micromanipulator and positioned above the dural surface. The position of the probe corresponded to a collateral blood-supplying area of 2–3 mm lateral to the midline, which is relatively devoid of large surface vessels (Coyle and Jokelainen, 1982). The exact site for probe positioning in the square was chosen so that the basal cerebral blood flow was between 100 and 150 PU. The Doppler-shift frequency range was chosen from 20 Hz to 12 kHz. The flow signal was averaged with a 0.2-s time constant. The analog output from the instrument was fed into a data acquisition/processing system (vide infra).

2.6. Experimental protocol

Peptides for i.v. and i.car. administration were freshly dissolved in saline (0.9% NaCl) or diluted from a frozen stock solution. After a 15 to 30-min stabilization period the peptides were infused in the doses indicated in Section 3 in a volume of 0.1 ml by means of a Braun infusion pump (Braun Perfusor) set at a rate of 500 μl/min over 12 s. In order to flush the cannula, each infusion of a peptide was followed by 0.05 ml saline for i.v. administration or heparin (50 IU/ml) for i.car. administration. Saline served as vehicle control.

In three series of experiments two successive dose–response curves were made for peptides being tested after i.v. and i.car. administration. One peptide was tested per rat. In the first and second series of experiments two dose–response curves were made after i.v. and i.car. administration with ligation of the side branches in each rat and blood pressure, heart rate, CF_{tot}, CF_{int} and cerebral blood flow were measured. In the third series of experiments dose–response curves were obtained after i.v. and

i.car. administration, or only after i.v. administration, without ligation of the side branches and blood pressure, heart rate, CF_{tot} and cerebral blood flow were measured.

The time interval between each dose of a peptide was 5–10 min, with 15 min between the different routes of administration in order to enable hemodynamics to recover completely.

2.7. Data acquisition and analysis

The hemodynamic signals (mean arterial pressure, heart rate, CF_{tot}, CF_{int}, and cerebral blood flow) were measured and processed by a data acquisition/processing system (a Bio Signal Processing System or Hemodynamic Acquisition System, Dept. of Instrumental Services, University of Limburg, Maastricht, Netherlands). The collected data were sent to a computer and mean arterial blood pressure, heart rate, mean CF_{tot} and CF_{int}, and cerebral blood flow were determined once every 500–1000 ms. Baseline values were calculated from the mean of a 30-s period before each administration. To determine the response to a peptide the mean for a 2-s period after administration was calculated.

Mean arterial pressure was calculated according to the formula: $(2 \times P_d + P_s)/3$, in which P_d is diastolic pressure and P_s systolic pressure.

To find dose–response relationships, the changes in mean arterial pressure, heart rate, mean CF_{tot} and CF_{int}, and cerebral blood flow were calculated at the time of maximal effect as compared to pre-injection values. The i.v. administration of saline had no effect on any hemodynamic variable. In some rats, however, i.car. administration of saline caused an increase in CF_{int} and cerebral blood flow. In order to calculate the true maximal change after administration of a peptide, CF_{int} and cerebral blood flow were corrected for the saline effect.

To calculate ED_{50} and E_{max} values a non-linear steepest gradient method was used to fit the data of the dose–response curves to the Hill equation: $E_D/E_{max} = [D^n/([ED_{50}]^n + [D]^n)]$, where E is the effect at dose D, E_{max} the (estimated) maximal effect at plateau, D the dose, and ED_{50} the effective dose to produce a half-maximal effect and n a measure of cooperativity. The ED_{50} and E_{max} are a measure of potency and intrinsic activity (α), respectively. Curves were fitted only according to this method when a plateau (E_{max}) level was reached.

The basal values for mean arterial pressure, heart rate, CF_{tot}, CF_{int}, and cerebral blood flow, and ED_{50} and E_{max} were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni test as post-hoc test. Data from the dose–response curves were analyzed by repeated-measures ANOVA followed by one-way ANOVA as post-hoc test. To compare ED_{50} and E_{max} after i.v. and i.car. administration an independent Student's t-test was used. Significance was set at the 95% probability level. All data are expressed as the means ± S.E.M.

2.8. Drugs

Heparin was purchased from Leo Pharmaceutical Products (Weesp, Netherlands; heparin sodium 5000 IU/ml) or Organon Technika (Boxtel, Netherlands; tromboliquin, heparin sodium, 5000 IU/ml) and urethane from Sigma (St. Louis, MO, USA). Lys-γ_2-MSH, γ-MSH-(4–12), γ-MSH-(5–12), and γ-MSH-(6–12) were synthesized at and kindly supplied by the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, Netherlands). ACTH-(4–10), ACTH-(4–9) and ACTH-(1–24) were a gift from Organon. γ_2-MSH and γ-MSH-(3–8) were purchased from Bachem (Bubendorf, Switzerland), and [Nle^4,D-Phe^7]α-MSH from Sigma.

The peptides were analyzed by fast atom bombardment mass spectrometry in the positive ion mode. In all cases, the protonated molecular ion (MH^+) was detected at the expected values of m/z (i.e., ±0.3 amu, $z = 1$).

3. Results

3.1. Effects of the 'mother compounds' Lys-γ_2-MSH, γ_2-MSH and ACTH-(4–10) on peripheral and central hemodynamics

In urethane-anesthetized rats both the naturally circulating peptide, Lys-γ_2-MSH, and the synthetic peptide, γ_2-MSH, caused, in most rats, a monophasic change in blood pressure following either i.v. or i.car. injection. However, a biphasic effect was seen occasionally. Therefore, the groups treated with γ-MSH (both natural and synthetic, $n = 14$ for each group) were divided into subgroups showing either a 'true pressor' or a 'monophasic depressor' or a 'biphasic' response. A 'true pressor' effect was defined as the response that caused a mean arterial pressure incraese of more than 15 mmHg for the highest dose of 100 nmol/kg. A 'true pressor' effect was observed for both peptides in 10 out of 14 rats, while mean arterial pressure decreased monophasically after i.v. administration in only one rat. A 'biphasic' response was observed in 5 out of 14 rats after administration of Lys-γ_2-MSH and in 2 out of 14 rats after administration of γ_2-MSH. Fig. 1 shows only the 'true pressor' responses. After i.car. injection of both peptides the 'true pressor' response was seen in all animals ($n = 14$) and only three animals in each group showed a biphasic response. No monophasic depressor responses were seen. The corticotropin analog, ACTH-(4–10), caused monophasic pressor responses only.

The effects of the three peptides on mean arterial blood pressure, heart rate and flow variables were qualitatively comparable whether administration was i.v. or i.car. The effects tended to be more potent after i.car. injection (a parallel shift of the dose–response curve over circa 0.5 log scale) than after i.v. administration, which became only highly significant for cerebral blood flow (dose × route of

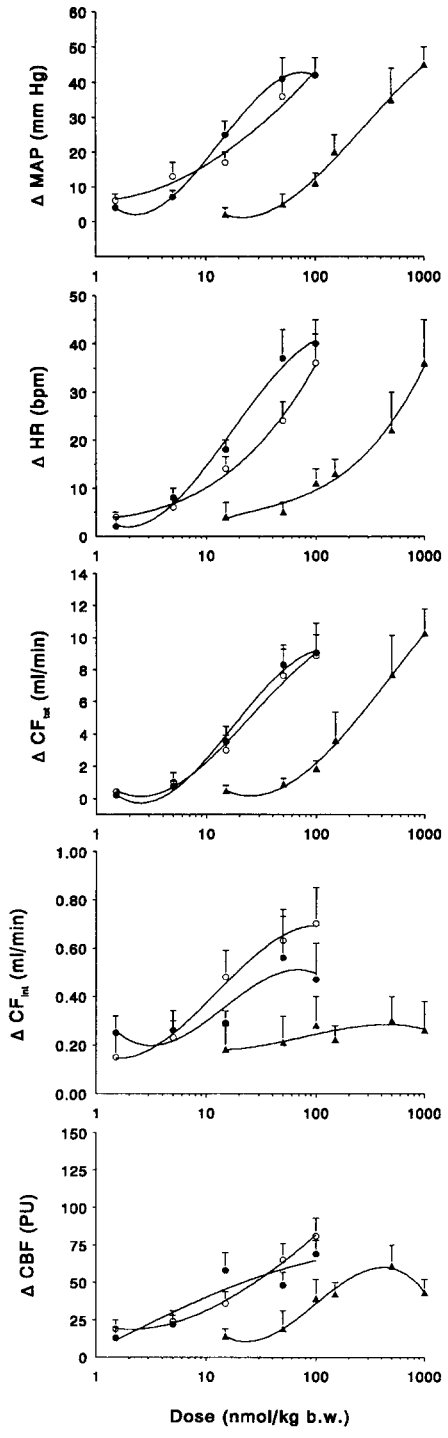

Fig. 1. Dose–response relationship for the 'mother compounds', Lys-γ_2-MSH (○),γ_2-MSH (●) and ACTH-(4–10) (▲), with respect to their effects on mean arterial pressure (MAP), heart rate (HR), total carotid artery blood flow (CF_{tot}), internal carotid artery blood flow (CF_{int}) and cortical cerebral blood flow (CBF) after i.car. administration to urethane-anesthetized rats. The results are expressed as absolute changes from pre-administration values and as means ± S.E.M. (n = 7–14).

administration interaction $F(3,66) = 3.19$, $P < 0.05$). Therefore, it was decided to present graphically only the results for i.car. injection. The pre-i.car. administration values for mean arterial pressure, heart rate, CF_{int} and cerebral blood flow for Lys-γ_2-MSH, γ_2-MSH and ACTH-(4–10) were not significantly different from each other (Table 2). Vehicle (saline) had no effect on either mean arterial pressure or heart rate. Vehicle (saline) had no significant effect on the flow variables after i.v. administration, but after i.car. administration a slight effect was seen in some rats. The effects of the different doses of the peptides were corrected for in these rats (see Section Section 2.7).

Both γ-MSH peptides and ACTH-(4–10) increased mean arterial pressure and heart rate dose dependently and significantly after i.v. (data not shown) and i.car. administration (Fig. 1) (mean arterial blood pressure: dose × treatment interaction $F(6,93) = 5.20$, $P < 0.05$) and heart rate: dose × treatment interaction $F(6,90) = 4.65$, $P < 0.05$), thereby showing similar potency and intrinsic activity (Table 3). The dose–response curve for ACTH-(4–10) was parallel to that for both γ-MSHs (Fig. 1) but with 10-fold lower potency ($P < 0.05$), although its efficacy was not different (Table 3).

Both i.v. (data not shown) and i.car. (Fig. 1) administration of the γ-MSHs and ACTH-(4–10) caused relatively strong and dose-dependent increases in CF_{tot} (i.car. administration: dose × treatment interaction $F(6,48) = 6.91$, $P < 0.05$) and cerebral blood flow (i.car. administration: dose × treatment interaction $F(6,81) = 2.42$, $P < 0.05$) (Fig. 1). The two γ-MSHs were about equipotent with respect to their effects on cerebral flow parameters, whereas ACTH-(4–10) was more than 5-fold less potent ($P < 0.05$; Table 4).

3.2. *Effects of structurally related γ-MSH analogs with N-terminal amino-acid chain shortening on peripheral and central hemodynamics*

In a second series of experiments the consequences of sequential removal of N-terminal amino acids from the γ-MSH structure were examined. The effects of three peptides were compared to the effects of γ_2-MSH: γ-MSH-(4–12), γ-MSH-(5–12) and the γ-MSH-(6–12) fragments. γ-MSH-(4–12) had a biphasic effect on blood pressure in three out of seven rats after i.v. and i.car. administration. The depressor component of this biphasic response was more pronounced after i.car. injection, with a maximal response of 15 ± 1 mmHg for a dose as low as 15 nmol/kg, than after i.v. administration, with a maximal response of 8 ± 3 mmHg for a dose of 100 nmol/kg. After i.v. administration of γ-MSH-(6–12) only a pressor response was observed, while i.car. injection was followed by a biphasic response with a depressor component of 17 ± 6 mmHg was observed in 3 out of 7 rats. After γ-MSH-(5–12) only a pressor response was seen following i.v. and i.car. administration. The effects of these three γ-MSHs on blood pressure (pressor response) were significantly more pronounced after i.car. administration (dose × route of administration interaction $F(5,100) = 2.66$, $P <$

Table 2
Pre-i.car. administration values of the various γ-MSH/ACTH peptides for the different cardiovascular parameters

	n	MAP (mmHg)	HR (bpm)	CF_{tot} (ml/min)	CF_{int} (ml/min)	CBF (PU)
Lys-γ_2-MSH	14	113 ± 4	410 ± 14	10.2 ± 0.8	2.1 ± 0.2	104 ± 5
γ_2-MSH	14	108 ± 5	406 ± 14	8.7 ± 0.9	1.8 ± 0.2	102 ± 4
γ-MSH-(4–12)	6	92 ± 3	407 ± 15	9.8 ± 1.0	1.1 ± 0.2	118 ± 13
γ-MSH-(5–12)	6	117 ± 7	376 ± 12	12.5 ± 1.3	1.6 ± 0.2	114 ± 9
γ-MSH-(6–12)	6	99 ± 6	438 ± 18	11.4 ± 1.0	1.4 ± 0.2	131 ± 7
ACTH-(4–10)	7	124 ± 5	374 ± 16	16.0 ± 1.0 [a]	2.1 ± 0.1	98 ± 4
ACTH-(1–24) [b]	6	91 ± 4	394 ± 14	8.4 ± 1.6	ND	122 ± 6
NDP-MSH [c]	3	97 ± 3	441 ± 21	5.8 ± 1.2	ND	125 ± 15

[a] Significantly different from Lys-γ_2-MSH and γ_2-MSH; [b] i.v. administration; [c] i.car. administration without ligation of the side branches; ND = not done.

0.05). Fig. 2 shows only the 'true pressor' responses for i.car. administration. The pre-i.car. administration values are given in Table 2 and were not significantly different between the various groups. Vehicle (saline) had no effect on either mean arterial pressure or heart rate. In case of the flow variables, vehicle (saline) had no significant effect after i.v. administration, whereas after i.car. administration a small effect was seen in some rats. The effects of the different doses of the peptides in these rats were corrected for (see Section 2.7).

Removal of the tripeptide, Tyr^1-Val^2-Met^3, which yields the fragment γ-MSH-(4–12), did not result in a pressor response significantly different from that observed following administration of γ_2-MSH (Fig. 2 and Table 3). Removal of the N-terminal tetrapeptide, Tyr^1-Val^2-Met^3-Gly^4 (γ-MSH-(5–12)) resulted in a significant loss of potency with the preservation of intrinsic activity, as shown by a shift to the right of the dose–response curve (Fig. 2 and Table 3). Further removal of His^5, yielding the γ-MSH-(6–12) fragment, restored the lost potency (Fig. 2 and Table 3). All three γ-MSH analogs caused a tachycardiac effect.

Fig. 2 and Table 4 show that the fragments γ-MSH-(4–12) and γ-MSH-(5–12) caused a highly significant (dose × treatment interaction $F(15,65) = 2.36$, $P < 0.05$) smaller increase in CF_{tot} as compared to that after γ_2-MSH. This loss in intrinsic activity was not found with γ-MSH-(6–12). With respect to the induced increases in CF_{int}, which reflects the macrocirculatory intracerebral blood flow, and cerebral blood flow, which reflects the microcirculatory cerebrocortical blood flow, no statistically significant difference was found between the effects of the four γ-MSHs.

3.3. Effects of structurally related γ-MSH and ACTH-(4–10) analogs with C-terminal amino-acid chain shortening on peripheral and central hemodynamics

In a third series of experiments the influence of a shortened C-terminus of γ-MSH by removal of the C-terminal tetrapeptide, Asp^9-Arg^{10}-Phe^{11}-Gly^{12} (γ-MSH-(3–8)), and of ACTH-(4–10) by removal of the amino acid, Gly^{10} (ACTH-(4–9)), was investigated. The i.v. (data not shown) and i.car. administration of Lys-γ_2-MSH (data not shown) and γ_2-MSH, in doses of 1.5–100 nmol/kg, caused significant and dose-dependent increases in mean arterial pressure and heart rate (Fig. 3). Following their i.v. administration to urethane-anesthetized rats ($n = 4$), the γ-MSH-(3–8) and ACTH-(4–9) fragments had no effect on mean arterial pressure, while there was a slight but significant ($P < 0.05$) increase in heart rate.

Following i.car. administration of γ-MSH (3–8) and ACTH-(4–9) fragments, only the latter fragment caused a

Table 3
Potency (ED_{50}) and intrinsic activity (E_{max}) of various γ-MSH/ACTH peptides with respect to mean arterial pressure and heart rate

	Mean arterial pressure				Heart rate			
	ED_{50} (nmol/kg)		ΔE_{max} (mmHg)		ED_{50} (nmol/kg)		ΔE_{max} (bpm)	
	i.v.	i.car.	i.v.	i.car.	i.v.	i.car.	i.v.	i.car.
Lys-γ_2-MSH	39 ± 10	19 ± 6	49 ± 8	40 ± 8	30 ± 8	30 ± 12	33 ± 9	37 ± 10
γ_2-MSH	26 ± 6	16 ± 3	49 ± 7	49 ± 8	30 ± 7	21 ± 5	55 ± 10	47 ± 9
γ-MSH-(4–12)	41 ± 7	22 ± 6	44 ± 4	45 ± 8	45 ± 27	13 ± 3	17 ± 8	23 ± 7
γ-MSH-(5–12)	113 ± 7 [a,b]	76 ± 32	43 ± 10	42 ± 8	75 ± 30	119 ± 50 [a,b]	29 ± 7	43 ± 5
γ-MSH-(6–12)	34 ± 5	14 ± 5 [c]	54 ± 4	58 ± 4	29 ± 7	25 ± 6	43 ± 9	40 ± 10
ACTH-(4–10)	NA	142 ± 17 [a,b]	NA	43 ± 8	NA	NA	NA	NA

Data are expressed as means ± S.E.M; [a] $P < 0.05$ significantly different from γ_2-MSH; [b] $P < 0.05$ significantly different from Lys-γ_2-MSH; [c] $P < 0.05$ significantly different from i.v. administration; NA = not available/incorrect fit.

Table 4
Potency (ED_{50}) and intrinsic activity (E_{max}) of various γ-MSH/ACTH peptides with respect to total carotid flow (CF_{tot}), internal carotid flow (CF_{int}), and cerebral blood flow (CBF)

	CF_{tot}				CF_{int}				CBF			
	ED_{50} (nmol/kg)		ΔE_{max} (ml/min)		ED_{50} (nmol/kg)		ΔE_{max} (ml/min)		ED_{50} (nmol/kg)		ΔE_{max} (PU)	
	i.v.	i.car.	i.v.	i.car.	i.v.	i.car.	i.v.	i.car.	i.v.	i.car.	i.v.	i.car.
Lys-γ_2-MSH	25 ± 1	37 ± 11	9.7 ± 4.1	10.9 ± 1.0	NA	18 ± 9	NA	0.8 ± 0.2	24 ± 9	23 ± 7	64 ± 9	79 ± 14
γ_2-MSH	21 ± 5	19 ± 5	9.0 ± 0.2	11.0 ± 1.7	NA	12 ± 9	NA	1.0 ± 0.3	25 ± 12	NA	73 ± 17	NA
γ-MSH-(4–12)	57 ± 6	9 ± 2 [c]	7.1 ± 1.0	5.7 ± 0.9	75 ± 14	22 ± 14	1.0 ± 0.2	0.6 ± 0.1	61 ± 14	13 ± 3 [c]	60 ± 14	72 ± 11
γ-MSH-(5–12)	388 ± 152	87 ± 43	5.1 ± 1.6	5.7 ± 2.1	100 ± 1	208 ± 76	1.1 ± 0.4	1.3 ± 0.1	323 ± 109 [a,b]	60 ± 32 [b,c]	61 ± 18	99 ± 28
γ-MSH-(6–12)	38 ± 3	25 ± 6	12.0 ± 5.2	11.0 ± 2.5	89 ± 11	13 ± 6 [c]	1.1 ± 0.2	0.5 ± 0.05	31 ± 6	14 ± 4	88 ± 10	81 ± 19
ACTH-(4–10)	NA	255 ± 50 [a,b]	NA	12.6 ± 1.3	NA	NA	NA	NA	NA	113 ± 29 [b]	NA	61 ± 5

Data are expressed as means ± S.E.M.; [a] $P < 0.05$ significantly different from γ_2-MSH; [b] $P < 0.05$ significantly different from Lys-γ_2-MSH; [c] $P < 0.05$ significantly different from i.v. administration; NA = not available/incorrect fit.

significant ($P < 0.05$) increase in mean arterial blood pressure and heart rate and, this, only at the highest dose (1000 nmol/kg body weight, $n = 4$) (Fig. 3).

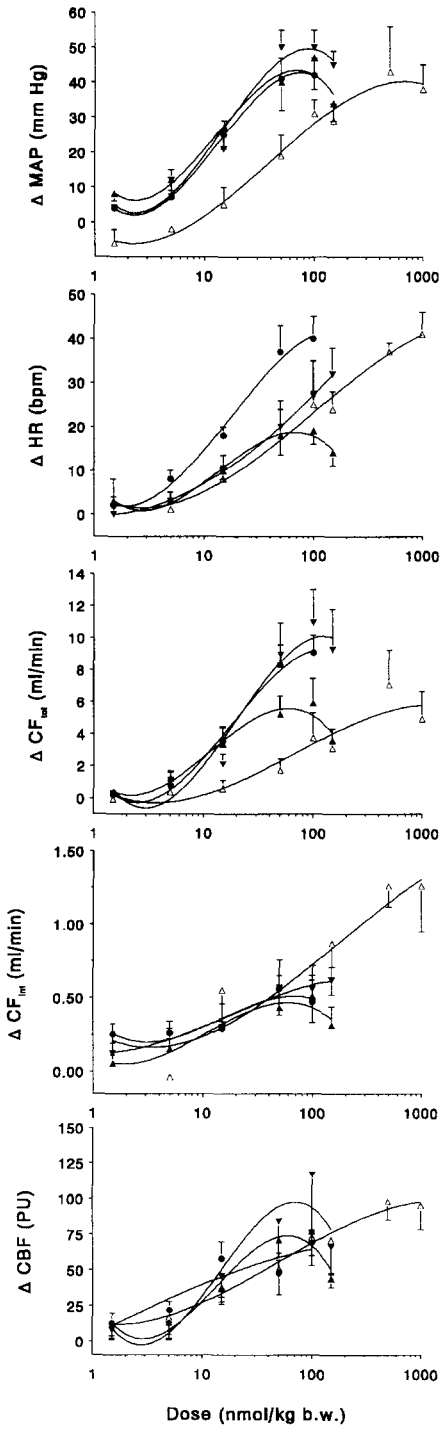

Fig. 2. Dose–response relationship for γ_2-MSH (●) and for γ-MSH analogs with N-terminal chain shortening (γ-MSH-(4–12), ▲; γ-MSH-(5–12), △; γ-MSH-(6–12), ▼) with respect to their effects on mean arterial blood pressure (MAP), heart rate (HR), total carotid blood flow (CF_{tot}), internal carotid blood flow (CF_{int}) and cortical cerebral blood flow (CBF) after i.car. administration to urethane-anesthetized rats. The results are expressed as absolute changes from pre-administration values and as means ± S.E.M. ($n = 6$–14).

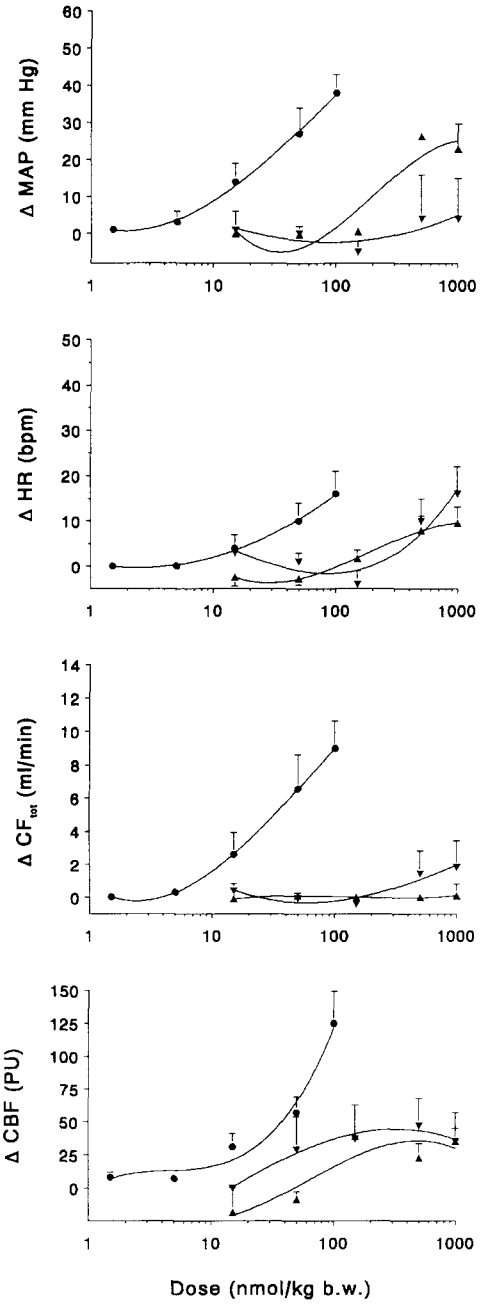

Fig. 3. Dose–response relationship for γ_2-MSH (●) and for γ-MSH/ACTH analogs with C-terminal shortening (γ-MSH-(3–8), ▼; ACTH-(4–9), ▲) with respect to their effects on mean arterial blood pressure (MAP), heart rate (HR), total carotid blood flow (CF_{tot}) and cortical cerebral blood flow (CBF) after i.car. administration to urethane-anesthetized rats. The results are expressed as absolute changes from pre-administration values and as means ± S.E.M. ($n = 4$–14).

The i.v. administration of both γ-MSH-(3–8) and ACTH-(4–9) produced no effects on CF_{tot} and cerebral blood flow (Fig. 3). The i.car. infusion of γ-MSH-(3–8) had no effect on CF_{tot} and cerebral blood flow, whereas ACTH-(4–9) caused only a slight but non-significant increase in cerebral blood flow without an effect on the CF_{tot} (Fig. 3).

3.4. Effects of NDP-MSH and ACTH-(1–24) on peripheral and central hemodynamics

In this same third series of experiments the effects of the stable α-MSH analog, NDP-MSH, and ACTH-(1–24) were studied on peripheral and central hemodynamics after i.v. administration. The i.v. administration of NDP-MSH had no significant effects on blood pressure (Fig. 4). The i.v. administration of ACTH-(1–24) produced a dose-dependent depressor effect (Fig. 4). After i.car. administration of NDP-MSH a significant ($P < 0.05$) depressor effect was observed for the highest dose (1000 nmol/kg), but there were no significant effects on cerebral blood flow. NDP-MSH and ACTH-(1–24) did not affect heart rate (Fig. 4). Both these peptides were without a significant effect on CF_{tot} and cerebral blood flow when injected i.v. (Fig. 4).

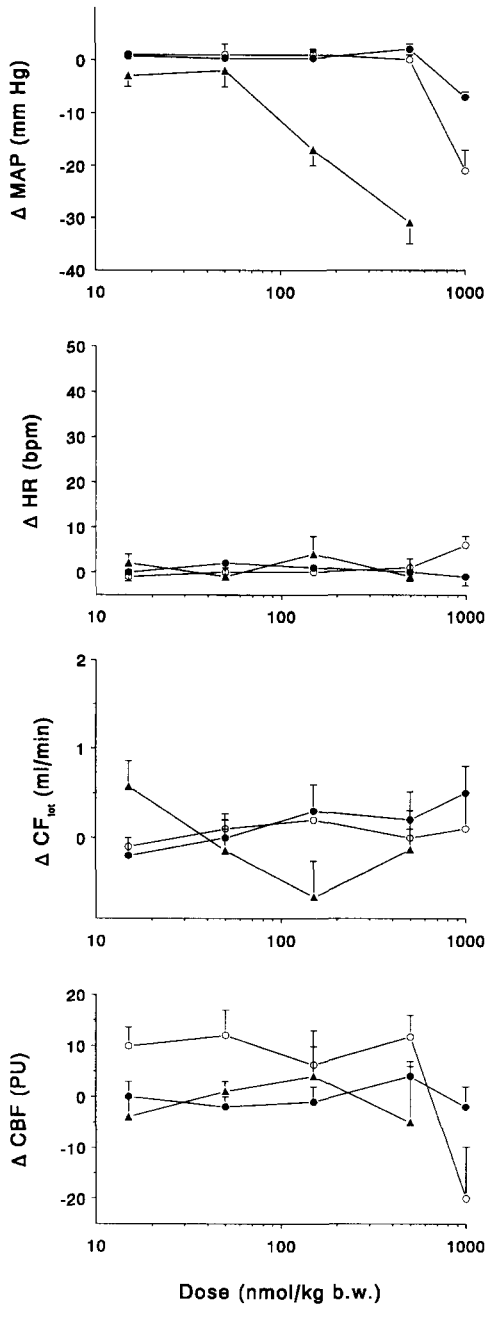

Fig. 4. Dose–response relationship for the stable α-MSH analog, NDP-MSH, and ACTH-(1–24) with respect to their effects on mean arterial blood pressure (MAP), heart rate (HR), total carotid blood flow (CF_{tot}) and cerebral cortical blood flow (CBF) after i.v. (NDP-MSH, ●; ACTH-(1–24), ▲) and i.car. (NDP-MSH, ○) administration to urethane-anesthetized rats. The results are expressed as absolute changes from the pre-administration values and as means ± S.E.M. ($n = 3$–6).

4. Discussion

It has been reported that the melanotropin, γ_2-MSH, and the partially homologous corticotropin, ACTH-(4–10), cause a short-lasting and pronounced increase in blood pressure and heart rate in both conscious (Gruber et al., 1984, 1985; Klein et al., 1985; Gruber and Callahan, 1989; Sun et al., 1992; De Wildt et al., 1993; Versteeg et al., 1993; Van Bergen et al., 1995) and anesthetized rats (De Wildt et al., 1993). Structure-activity analysis of γ-MSH- and ACTH-like peptides in conscious rats with respect to their effects on peripheral hemodynamic parameters revealed that C-terminal shortening of γ_2-MSH and of ACTH-(4–10) results in a loss of intrinsic activity (E_{max}) (Van Bergen et al., 1995). Based on these results, we postulated that the intrinsic activity of γ_2-MSH and of ACTH-(4–10) is carried by the C-terminal sequence, Asp^9-Arg^{10}-Phe^{11} and Gly^{10}, respectively (Van Bergen et al., 1995). Removal of amino acids from the N-terminal part of γ_2-MSH and ACTH-(4–10) had consequences only for the potency (ED_{50} values), i.e., for the affinity for the postulated melanocortin receptor. The minimal sequence, and thus the message site within the molecule, is supposed to reside in the γ-MSH/ACTH fragment, His-Phe-Arg-Trp, or in the even shorter fragment, Phe-Arg-Trp (Van Bergen et al., 1995). The structurally related POMC-derived peptides, α-MSH (De Wildt et al., 1993) and its stable analog, NDP-MSH, and ACTH-(1–24) (Van Bergen et al., submitted) appeared to be devoid of pressor effects, while the latter peptide induces a depressor response in combination with a reflex tachycardia in rats (Nakamura et al., 1976; Van Bergen et al., submitted) and rabbits (Szabo et al., 1987; Ludbrook and Ventura, 1995).

Recently, we reported that, in addition to its peripheral hemodynamic effects, γ_2-MSH has a pronounced influence on cerebral hemodynamics in urethane-anesthetized rats (De Wildt et al., 1995). The peptide was able to markedly increase both extracranial and intracerebral blood flow, indicative of increased blood perfusion to the brain, which could be explained at least partly by a direct cerebrovasodilating action of this peptide apart from its pressor and therefore perfusion pressure elevating action (De Wildt et al., 1995). Also, the regional microcirculatory cerebrocortical blood flow was reproducibly increased after adminis-

tration of this melanotropin, an effect which also appeared to be centrally mediated (De Wildt et al., 1995).

In the present study we extended the structure-activity analysis of γ-MSH/ACTH-like peptides to their effects on cerebral hemodynamics in urethane-anesthetized rats, in order to determine whether the sequence in the γ_2-MSH/ACTH-(4–10) molecule which is determinant for selectivity for peripheral cardiovascular actions, is also crucial for the central hemodynamic features of the peptides. Since it had been shown that, with γ_2-MSH, that the peptide-induced peripheral (De Wildt et al., 1995; Callahan et al., 1988) and cerebral (De Wildt et al., 1995) hemodynamic effects were more pronounced after i.car. administration (i.e., administration into a direct blood supply to the forebrain), than after i.v. infusion, we performed experiments with both routes of administration for the structural analogs. Indeed, as can be seen from Table 3, the hemodynamic effects of the various fragments were generally more pronounced after i.car. administration than after i.v. administration.

The strong peripheral cardiovascular (pressor and tachycardiac) effects now shown for γ_2-MSH, its naturally occurring analog, Lys-γ_2-MSH, and ACTH-(4–10) in urethane-anesthetized rats were consistent with those reported previously for conscious rats (Callahan et al., 1984; Klein et al., 1985; De Wildt et al., 1993; Versteeg et al., 1993; Van Bergen et al., 1995). Although the pressor and tachycardiac response appears to be somewhat less pronounced in anesthetized animals, the data for the urethane-anesthetized rats did not differ essentially from those for conscious animals. For this reason we could consider the urethane-anesthetized rat a useful model to investigate effects of the peptides on cerebral hemodynamics. In accordance with previous observations concerning the time course of the blood pressure response after i.v. applied γ_2-MSH in urethane-anesthetized rats (De Wildt et al., 1993), the present study showed that there is a tendency to develop a depressor effect in the case of i.v. administered Lys-γ_2-MSH and the γ-MSH-(4–12) analog. In most cases the depressor effect is immediately overridden by the pressor response (biphasic response); only in a few cases did the response appear to be monophasic. The occurrence of a rather strong depressor effect after i.car. Lys-γ_2-MSH, γ-MSH-(4–12) and γ-MSH-(6–12) might be indicative of a centrally mediated depressor action of these peptides. Such an assumption is in line with a previously observed depressor action of γ_2-MSH, which has been explained as the consequence of an interaction of the melanotropin with structures within the brainstem, i.e., the nucleus tractus solitarii, since injection of γ_2-MSH in this region induces a strong depressor action (De Wildt et al., 1994). After i.v. and, in particular, after i.car. administration of the peptides higher brain structures (e.g., the hypothalamus), which are involved in the pressor action, are also reached. As previously observed for γ_2-MSH (De Wildt et al., 1995), both Lys-γ_2-MSH and ACTH-(4–10) caused a relatively strong increase (> 100%) in CF_{tot}, implying an increase in extracranial and/or intracerebral blood flow. Measurement of CF_{int} indicates that a part of the increase in CF_{tot} can be ascribed to an augmented intracerebral blood flow. ACTH-(4–10) appears to be less efficacious than the two melanotropic compounds in this respect. Regarding the microcirculation in the cerebral cortex as reflected by cerebral blood flow, the melanotropic peptides and the corticotropin had a comparable intrinsic activity. The potencies (ED_{50} values) for γ_2-MSH and its naturally occurring derivative were similar, whereas the potency of ACTH-(4–10) was about 10-fold lower than that of the two melanotropins. At first glance the same order of potencies for the effect of the three peptides regarding blood pressure and heart rate on the one hand and cerebral hemodynamic parameters on the other, might indicate conformity between mechanisms underlying either the peripheral or central hemodynamic effects mediated by these key peptides. However, further analysis of the peripheral and central hemodynamic effects brought about by the N-terminal amino-acid shortened fragments suggests dissociated mechanisms.

Stepwise N-terminal chain shortening of γ_2-MSH to the γ-MSH fragments, γ-MSH-(4–12) and γ-MSH-(5–12) and γ-MSH-(6–12), resulted in a pharmacological profile for the peripheral cardiovascular parameters (blood pressure and heart rate) in the urethane-anesthetized rats comparable to that observed in our previous study in conscious rats (Van Bergen et al., 1995). Removal of Tyr^1-Val^2-Met^3, resulting in γ-MSH-(4–12), had no influence on potency, while further shortening by removal of the Gly^4 residue resulted in a peptide with a lower potency without affecting efficacy. As observed in conscious animals, removal of an additional residue, His^5, surprisingly did not lead to a further reduction, but to full restoration, of potency. We explained this as being the result of hindrance by the Arg^7-hydrophobic sequence by the His^5 residue (Van Bergen et al., 1995). Since His^5 is not lipophilic, only Arg^{10}-Phe^{11} at the C-terminal site can account for the pressor and tachycardiac effects. Removal of His^5 unmasks the second Arg^7-hydrophobic sequence: the presence of both amino acids surrounding Arg^7 (Phe^6 and Trp^8) now makes γ-MSH-(6–12) highly lipophilic. This could explain the increased potency of this γ-MSH fragment. However, a different pattern regarding central hemodynamics was observed for the three fragments from which N-terminal amino acids were removed. The efficacy to increase CF_{tot} was substantially smaller for γ-MSH-(4–12) and γ-MSH-(5–12) than for γ_2-MSH. However, there were no significant differences in intrinsic activity and potency with respect to the intracerebral hemodynamic parameters (CF_{int} and cerebral blood flow) for these fragments in comparison with the mother compound. So, there appears to be a difference in the rank order of efficacy and potency for the peripheral effects on the one hand and the central hemodynamic effects on the other, which suggests that different mechanisms (e.g., site of actions and/or

receptors) are involved in the two types of effects. Furthermore, it should be noted that the increase in CF_{tot} by γ-MSH fragments cannot not be solely dependent on the induced increase in blood pressure (c.q., perfusion pressure) since equal effectiveness on blood pressure for the γ-MSHs was not paralleled by the increase in CF_{tot}. Therefore, an active vasodilatory effect caused by γ-MSHs must be assumed to occur intracerebrally and/or within the head-neck region. Considering the central hemodynamic effects, again, the removal of His^5 appears to be crucial for the full expression of the cerebral flow effects of γ-MSH-(6–12).

In accordance with previous results obtained in conscious rats (Van Bergen et al., 1995), the absence of effects after C-terminal shortening of γ_2-MSH (γ-MSH-(3–8)) and ACTH-(4–10) (ACTH-(4–9)) on both peripheral and central hemodynamics in urethane-anesthetized rats in this study suggests that the intrinsic cardiovascular activity of γ-MSH and ACTH-(4–10) is carried by the C-terminal fragment Asp^9-Arg^{10}-Phe^{11} and Gly^{10}, respectively.

Neither NDP-MSH nor ACTH-(1–24) were able to produce an increase in blood pressure, as did some of the above mentioned structurally related melanotropins and corticotropins in urethane-anesthetized rats. In line with previous results in conscious rats (Van Bergen, submitted), ACTH-(1–24) and, to a lesser extent, NDP-MSH caused a depressor response in the anesthetized rats. It might be speculated that this depressor action can be related with the biphasic or monophasic hypotensive effect seen for some of the melanocortin peptides, as was described above. The origin of this depressor effect might be located within the brainstem, e.g., the nucleus tractus solitarii (vide supra).

In the early 90s five subtypes of the melanocortin receptor were cloned and identified (for a review, see Low et al., 1994). In vitro studies have demonstrated that γ_2-MSH has affinity for, and can activate, the melanocortin MC_3 receptor, while it has a substantially lower affinity for the other melanocortin receptor subtypes (Gantz et al., 1993; Roselli-Rehfuss et al., 1993; see Low et al., 1994). The melanocortin MC_3 receptor is localized in brain areas which are involved in cardiovascular regulation (Roselli-Rehfuss et al., 1993; for a review, see Low et al., 1994). Therefore, it can be hypothesized that the melanocortin MC_3 receptor is involved in mediating the peripheral (pressor and tachycardiac) as well as the cerebral (macro- and microcirculatory blood flow enhancing) hemodynamic effects of γ_2-MSH. However, the absence of any pressor or cerebral flow enhancing effects of NDP-MSH, a non-selective agonist for melanocortin receptors with a high affinity for the MC_1, MC_4 and MC_5 subtypes of the melanocortin receptor and which binds and activates the melanocortin MC_3 receptor with the same order of potency as does γ_2-MSH (Mountjoy et al., 1992, 1994; Gantz et al., 1993; Chhajlani et al., 1993), leads us to reject this hypothesis. Furthermore, the findings with the corticotropin, ACTH-(1–24), which is an agonist for the melanocortin MC_2 receptor (Mountjoy et al., 1992), exclude a role for this subtype of receptor in the peripheral and central hemodynamic effects of γ_2-MSH and related analogs as well. Discrepancies in structure-activity relationship of γ-MSH/ACTH-like peptides regarding their effects on blood pressure in conscious rats (Van Bergen et al., 1995) and on peripheral and central hemodynamics in anesthetized rats (present study) with the pharmacological profile of the receptors in vitro (Roselli-Rehfuss et al., 1993; Adan et al., 1994; Sahm et al., 1994) lead us to postulate that an as yet unidentified subtype of the melanocortin receptor is involved in the cardiovascular effects of the γ-MSHs. A clue for the elucidation of this receptor might be found in the structural features of the amino-acid sequence of the N-terminally shortened γ-MSHs. Namely, the γ-MSH-(6–12) sequence appeared to be a very important determinant for the effectiveness to alter peripheral and central hemodynamics. Of all the peptides studied, this γ-MSH fragment is the sequence which has the highest potency and efficacy with respect to peripheral and central hemodynamic actions. Further investigations should be directed towards finding a subtype of melanocortin receptor for which this fragment displays high selectivity as an agonist.

In addition to contributing to the possible discovery of a melanocortin receptor subtype specifically involved in hemodynamic processes, this key peptide, γ-MSH-(6–12), might give further support to the neuropeptide concept as it was formulated by De Wied (1969). This concept comprises the hypothesis that peptides derived from pro-opiomelanocortin (ACTH, α-MSH, endorphins and other peptides) can have central nervous system activities, such as profound effects on behavior, occurring independently of the classical peripheral endocrine effects. It appeared that (fragments of) these peptides are also produced by neurons from the same precursor molecule, POMC, which causes them to be defined as neuropeptides (De Wied, 1969). After processing of the POMC molecule in the various cerebral neurons the brain often uses smaller fragments of neuropeptides which originate from this precursor molecule as messages for distinct functions (De Wied, 1969). The present structure-activity analysis with the γ-MSH/ACTH fragments unequivocally places the locus of the hemodynamic activity in the 6–12 sequence of the γ-MSHs. Further studies on gene expression and post-translational enzymatic processing of the POMC molecule to final cleavage products such as γ-MSH-(6–12) could offer new leads to answer these questions.

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