Effects of tourniquet-induced ischemia on the release of proopiomedullin in peripheral blood plasma

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Proopiomedullin (POMC) derivatives such as β-endorphin have been reported to be released into the cardiovascular system during physical exercise (10, 20, 22, 27), which has been attributed to the stimulatory effect of elevated concentrations of lactate (3, 21), acidosis (10, 32), or an alteration of base excess. In five of eight patients, we found a significant increase of β-endorphin IRM, authentic β-endorphin, adrenocorticotropin, lactate, pH, and base excess. In eight patients undergoing knee arthroplasty under spinal anesthesia, catheters were inserted into the femoral vein proximally to thigh tourniquet location. Blood was drawn from these catheters 5 min before and 40 s, 5 min, and 10 min after tourniquet deflation to measure plasma concentrations of N-acetyl-β-endorphin immunoreactive material (IRM), β-endorphin IRM, authentic β-endorphin, adrenocorticotropic, lactate, pH, and base excess. In five of eight patients, we found a significant increase of β-endorphin IRM levels 40 s after tourniquet deflation compared with predemotion levels; 5 and 10 min after tourniquet deflation, the β-endorphin IRM levels were below the detection limit. Thus β-endorphin IRM was released from ischemic limb tissues into the cardiovascular system. Only a small part of the determined β-endorphin IRM corresponded to authentic β-endorphin. Forty seconds after tourniquet deflation, the β-endorphin IRM concentration correlated with base excess (r < 0.71; P < 0.05); no significant correlations were found with pH or lactate levels. Thus it was shown here for the first time that ischmic stress may induce the release of β-endorphin IRM from nonpituitary tissues.

Functional significance of proopiomedullin fragments in plasma; authentic β-endorphin in plasma; base excess; corticotroph- or melanotroph-type proopiomedullin systems

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tissues was obtained through a catheter inserted into the femoral vein in a position proximal to the thigh tourniquet and was analyzed for POMC derivatives, lactate, and blood-gas parameters.

In the plasma from the reperfusion blood of eight patients undergoing knee arthroplasty under spinal anesthesia, we determined the concentrations of N-acetyl-β-endorphin immunoreactive material (IRM), β-endorphin IRM, authentic β-endorphin [β-endorphin(1–31)], and ACTH, as well as pH, BE, and lactate concentration 5 min before (tA), as well as 40 s (tB), 5 min (tc), and 10 min (tD) after tourniquet deflation.

METHODS

Patients

The prospective study was approved by the Ethics Committee of the University of Giessen (Medical Faculty). Written consent to participate in the study was given by eight patients (see Table 1 for characteristics) who were all undergoing elective knee arthroplasty. Exclusion criteria were chronic painful syndromes, endocrinological disorders, pituitary insufficiency, perioperative use of opioids or hormones such as corticosteroids, and contraindications against a spinal anesthesia.

Preliminary Experiments

Preliminary experiments (n = 3) were conducted to assess the effects of tourniquet application on pH and lactate concentrations in reperfusion blood, drawn from the catheter inserted into the vena femoralis (V. femoralis catheter) proximally to the tourniquet position on the leg, which required surgical intervention. To assess the point of time t0 (maximum of lactate concentration, minimum of pH), 2 ml of blood were drawn from the V. femoralis catheter to determine lactate concentration and blood-gas parameters 10 s before, at the moment of tourniquet deflation, and every 10 s (until 120 s) after tourniquet deflation, as well as 3, 4, and 5 min thereafter.

Surgical and Anesthesiological Procedures

All patients were premedicated with midazolam (7.5 mg/os) 30 min before induction of anesthesia. After 500 ml of Ringer’s solution was administered into a forearm or dorsal hand vein, all patients were placed in a sitting position. Prilocaine (1%) for local anesthesia was administered, and a 25-gauge spinal needle (Spinocan, B. Braun, Melsungen, Germany) was inserted at the L3–L4 intervertebral level and advanced into the subarachnoid space, and 0.5% bupivacaine was injected. A venous catheter (V. femoralis catheter; Hydrocathe, 18 gauge, 20 cm, Becton Dickinson, Singapore) was inserted into the femoral vein ~4 cm below the ligamentum inguinale on the leg subjected to pneumatic tourniquet before surgery. In addition to spinal anesthesia, patients received 1% propofol (2,6-diisopropylphenol; 1.31–10.42 mg·kg body wt·h−1·h−1) as a hypnotic, which allowed spontaneous respiration during the operation. A pneumatic thigh tourniquet was applied as closely as possible below the V. femoralis catheter; the tourniquet pressure was kept between 350 and 400 mmHg. Surgical procedures started after tourniquet inflation; the mean duration of tourniquet-generated ischemia was 122 ± 10 min. At tA, tB, tc, and tD, venous blood was taken from the V. femoralis catheter.

Plasma Sample Collection

Twelve milliliters of blood were taken from the V. femoralis catheter, mixed with 150 μl of EDTA (0.08 g/ml), immediately placed on ice, and centrifuged at 1.000 g (15 min at 4°C). Approximately 1 ml of plasma was used for determination of ACTH. Two 5-ml plasma aliquots were acidified with 0.5 m HCl (1 M) each. All samples were immediately frozen and stored at −20°C until extraction. For assay standardization, β-endorphin(1–31) or N-acetyl-β-endorphin was added to EDTA plasma samples from three healthy volunteers in concentrations of 0.33, 1, 3.3, 10, 33, 100, and 1,000 pmol/l. These plasma standard samples were treated like test samples.

An additional 2 ml of blood were drawn from the V. femoralis catheter to measure lactate concentration, pH, and BE. These blood samples were collected in prechilled heparinized 3-ml blood-gas syringes and transferred to prechilled 2-ml centrifuge tubes; analysis was performed within 2 min after collection using a lactate and blood-gas analyzer (Profile Ultra, Nova Biomedical, Rödermark, Germany). Each sample was analyzed in duplicate. In the blood-gas analyzer, the standard BE in the extracellular fluid (ECF) was calculated as already described (19) and is given by

$$BE_{ECF} = C_{HCO_3^-} - 24.8 \frac{mmol}{l} + 16.2 (pH - 7.4) \frac{mmol}{l} \quad (1)$$

where HCO3− concentration (C_{HCO_3^-}) and BE are given in mM.

Plasma Extraction

The extraction of test and standard plasma samples was conducted as described previously (34). In brief, the samples were thawed at 4°C and centrifuged (5 min, 8,000 g), and 5-ml acidified plasma aliquots were passed at 4°C through Sep-Pak C18 cartridges (Waters), which had been activated before with 5 ml of methanol followed by 5 ml of water (bidistilled, 4°C). Then the cartridges were washed with 10 ml of acetic acid (4% in water) and 10 ml of water. Elution of the POMC derivatives from the cartridge was achieved with 10 ml of 1-propanol with 4% acetic acid (96/4 = vol/vol). The organic solvent contained in the eluate was evaporated at room temperature using a Speed Vac concentrator (Savant). The remaining aqueous phase was lyophilized, the residue was reconstituted on ice in 0.5 ml of buffer 1 (see Determination of POMC Fragments), and 100-μl aliquots [referred to as “extracts” (see Determination of POMC Fragments)] were frozen at −20°C and kept for further analyses.

Table 1. Demographic and clinical data of 8 patients undergoing knee arthroplasty

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Minimum</th>
<th>First Quartile</th>
<th>Second Quartile (Median)</th>
<th>Third Quartile</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male:female</td>
<td>3:5</td>
<td>50</td>
<td>60</td>
<td>65.5</td>
<td>69</td>
</tr>
<tr>
<td>Age, yr</td>
<td>50</td>
<td>60</td>
<td>65.5</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>ASA (II:III)</td>
<td>7:1</td>
<td>24.6</td>
<td>28.9</td>
<td>33.5</td>
<td>36.8</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>158</td>
<td>161</td>
<td>172</td>
<td>181.5</td>
<td>190</td>
</tr>
<tr>
<td>Surgery</td>
<td>108</td>
<td>116</td>
<td>120.5</td>
<td>129</td>
<td>139</td>
</tr>
</tbody>
</table>

ASA, American Society of Anesthesiologists classification.

J Appl Physiol • VOL 97 • SEPTEMBER 2004 • www.jap.org
Determination of POMC Fragments

Peptides, reagents, and buffer. Synthetic human β-endorphin(1-31) and N-acetyl-β-endorphin(1-31) were obtained from Bachem, Heidelberg, Germany, or from Novo-Biochem, Bad Soden, Germany, respectively. A monoclonal antibody against the NH₂-terminus of β-endorphin, code 3E7, was obtained from C. Gramsch, Schwab-hausen, Germany. Buffers used were

- 0.01% (wt/vol) bovine serum albumin, and 0.01% (wt/vol) thimerosal
- 0.1% (vol/vol) Triton X-100.

German. Buffers used were

- 0.1% (vol/vol) sodium chloride, 0.1% (wt/vol) gelatin, 0.05 M sodium phosphate (pH = 7.40) containing 0.15 mol/ml sodium chloride, 0.1% (wt/vol) bovine serum albumin, and 0.01% (wt/vol) thimerosal and buffer 2, which consisted of buffer 1 with an additional 0.1% (vol/vol) Triton X-100.

β-Endorphin(1-31). A fluid phase two-site immunoprecipitation RIA as described previously was used (12) to determinate β-endorphin(1-31) in plasma extracts. In brief, a monoclonal mouse antibody (code 3E7), directed against the NH₂-terminus of β-endorphin, and a polyclonal rabbit antiserum (code 11P), directed against the COOH-terminal fragment of β-endorphin, were used. The monoclonal mouse antibody (3E7) was radioactively labeled with 125I by using published techniques (15, 34); for purification, the iodination product was incubated with charcoal for 15 min on ice, the suspension was centrifuged at 8,000 g (5 min), and the supernatant containing the 125I-labeled antibody (3E7) was stored at 4°C.

For the assay, plasma extracts were thawed at 4°C and incubated together with the labeled antibody (code 3E7) and rabbit antiserum (code 11P). A [3H]-3E7-β-endorphin/125I complex was formed in case of samples containing β-endorphin(1-31). The complex was immunoprecipitated by a goat antiserum directed against rabbit immunoglobulin. The immunoprecipitate was centrifuged; the radioactive activity measured in the pellet indicated the amount of precipitated β-endorphin(1-31).

This two-site immunoprecipitation RIA proved highly specific for β-endorphin(1-31). β-Lipotropin, fragments of β-endorphin, e.g., α- or γ-endorphin, N-acetylated endorphins, Met-enkephalin, or all other peptides tested thus far did not cross-react with this assay. The intra-assay and interassay coefficients of variation were 3.7 or 3.8%, respectively. The detection limit varied between 2 and 5 pmol β-endorphin(1-31)/liter plasma and was determined separately for each assay (12, 27).

β-Endorphin IRM. β-Endorphin(17-26) IRM in the plasma extracts was determined in a one-site fluid-phase RIA as described in principle (34) and recently characterized (12). In brief, residues from lyophilization were reconstituted in buffer 1 on ice, and aliquots thereof were incubated together with a polyclonal rabbit antiserum (code 84E) against the 17-26 fragment of human β-endorphin (diluted 1:10,000 with buffer 1) and with 125I-labeled β-endorphin(1-31) in buffer 2 at 4°C for 24 h. To separate antibody-bound from free-labeled peptide, the samples were incubated together with charcoal and subsequently centrifuged to remove charcoal-adsorbed peptide. The supernatants containing antibody-bound labeled and nonlabeled peptide in amounts dependent on the amount of nonlabeled peptide in the samples were analyzed for radioactivity in a gamma counter to determine antibody-bound labeled peptide.

N-acetyl-β-endorphin IRM. N-acetyl-β-endorphin IRM was determined using a competitive fluid-phase RIA, which was conducted according to previously described assays (34) and will be published elsewhere in detail. In brief, plasma extracts (in buffer 1) were thawed at 4°C and were incubated together with a polyclonal rabbit antiserum (code 27P) against the acetylated NH₂-terminus of β-endorphin and with 125I-labeled N-acetyl-β-endorphin(1-27) (in buffer 2) at 4°C for 24 h (for further conduction of the assay, see β-Endorphin IRM). The assay picks up all N-acetylated β-endorphin derivatives but does not recognize nonacylated derivatives. The detection limit was 3.5 pmol/l plasma, the intra-assay coefficient of variation was 3.6%, and the interassay coefficient of variation was 4.3%.

ACTH. ACTH(1-39) in plasma was measured by a commercially available two-site chemiluminescence assay (Nichols, San Juan Capistrano, CA). The detection limit was 0.5 ng/l.

Statistical Analysis

For descriptive statistics, minimum and maximum values as well as first, second (median), and third quartiles, e.g., of POMC-derivative plasma concentrations, were calculated. Analytical statistics were performed with the Wilcoxon’s signed rank test for nonparametric data. The significance of differences between plasma concentrations of POMC derivatives at different times was examined by calculating Hodges-Lehmann estimators (θ) associated with Wilcoxon’s signed rank statistics (13); the level of significance was set at the two-sided 95% confidence interval for θ. To estimate differences between these concentrations, Hodges-Lehmann estimators, θ1/2A, θ1/2B, and θ1/2C as well as θA–D or θB–D were calculated. Concentration differences deviating significantly from zero are indicated by a 95% confidence interval limited by two negative or two positive values; one of the two values is also allowed to be zero. Spearman’s rank correlation coefficients were calculated for analysis of interrelationships between plasma concentrations of POMC derivatives and metabolic parameters like acidosis, alteration of BE, or lactate concentrations at tA, tB, tC, or tD.

RESULTS

The results of the preliminary experiments (n = 3) are given in Fig. 1. Maximal lactate concentrations were found between 40 and 80 s; the minimum of blood pH was found at 40 s after tourniquet deflation. Thus we set the point of tB in our study at 40 s after tourniquet deflation.

Considering distribution-free descriptive statistics, the first quartiles, the medians, and the third quartiles of the plasma concentrations of β-endorphin IRM, β-endorphin(1-31), N-acetyl-β-endorphin IRM, ACTH, and lactate in reperfusion blood were calculated (Table 2). Only a small part of the determined β-endorphin IRM corresponded to β-endorphin(1-31). With the exception of three samples, the concentrations of β-endorphin(1-31) after tourniquet deflation were below the detection limit. In reperfusion blood, only low plasma concentrations of N-acetyl-β-endorphin IRM without any significant change were observed.

J Appl Physiol • VOL 97 • SEPTEMBER 2004 • www.jap.org
DISCUSSION

The data of ACTH and β-endorphin IRM listed in Table 2 reflect different reactions of the corticotroph-type POMC system to the perioperative conditions. On the one hand, plasma levels of ACTH did not increase significantly after tourniquet deflation (from tA to tB), but later, from tC to tD, there was a significant increase (10 min after tourniquet deflation). On the other hand, we observed a significant increase of β-endorphin IRM plasma levels at tB compared with the levels at tA, which were below the detection limit in all patients and were thus assumed to be basic levels. At tC and tD, the concentrations of β-endorphin IRM in the reperfusion blood plasma were again below the detection limit (Fig. 2).

The levels of β-endorphin IRM and ACTH determined in reperfusion blood plasma have been condensed to box-and-whisker plots of minimum and maximum values as well as first, second (medians), and third quartiles of the concentrations (Fig. 2). In addition, the significance of the concentration differences was tested using Wilcoxon’s matched-pairs signed rank test (Fig. 2).

Metabolic changes as observed after tourniquet deflation in reperfusion-blood, i.e., pH, BE in the ECF, are shown in Table 2. Levels of lactate and the BE increased, and pH decreased significantly (from tA to tB) in the reperfusion blood after a period of ischaemia (Table 2).

At tB, a significant correlation between the alteration of the BE in the ECF [ΔBE(tB−tA) = BE(tB) − BE(tA)], and the alteration of β-endorphin IRM concentrations (C) [ΔCβ-endorphin IRM(tB−tA)] in the reperfusion blood in relation to the basic levels at tA was found [rβ-endorphin IRM(tB−tA), ΔBE(tB−tA)] = 0.71, P < 0.05); in contrast, we detected no correlation between the alteration of β-endorphin IRM levels and the alteration of pH and lactate concentrations. No correlation was found between the release of β-endorphin IRM and the BE in blood cells, nor was any correlation observed between the change in PO2 or PCO2 or the alteration of concentrations in the examined POMC derivatives in the reperfusion blood.

Metabolic changes during physical exercise are associated with high levels of POMC derivatives such as ACTH or
β-endorphin determined in venous blood plasma. Apparently, the behavior of β-endorphin is not directly dependent on exercise intensity, leaving the question for a parameter more closely related to changes in β-endorphin concentrations during exercise. Earlier studies demonstrated a relationship between high levels of lactate and β-endorphin release (3, 7), a finding not supported by subsequent investigators (24); later results suggested that the β-endorphin release during exercise is a simple function of the level of metabolic acidosis (10, 28, 32). Subsequently, a strong relationship between BE and β-endorphin concentrations during exercise has been found (32). However, the origin of the POMC derivatives determined in venous blood as well as the exact mechanisms responsible for the increase of POMC derivative concentrations is not yet known. Considering that anaerobic metabolic changes under a state of ischemia have an influence on the whole organism and not only on the hypothalamic-pituitary-adrenal axis, we investigated the influence of anaerobic metabolic changes, such as acidosis, alteration of lactate concentration, BE, or PCO2 and Po2 on the release of POMC derivatives into the venous blood of an extremity on tourniquet-induced ischaemia.

Peripheral Source of POMC Derivatives Determined in Blood Plasma

In fact, at t0, the β-endorphin IRM concentration in reperfusion blood drawn from the V. femoralis catheter, increased in relation to the basal pretourniquet deflation levels. The time lag between tourniquet deflation and β-endorphin response (40 s) is not consistent with the known response latency of β-endorphin release observed on activation of the hypothalamic-pituitary-adrenal axis to various kinds of stress (~3–5 min) (18, 31). Therefore, we conclude that the β-endorphin IRM determined in the reperfusion blood at t0 is not released from the pituitary but from tissues in the ischemic limb. This would be compatible with the expression of POMC in a number of tissues of the mammalian organism; POMC-processing cells have been detected in various nonpituitary tissues (17), e.g., human dermal microvascular endothelial cells, which express POMC, and PC-1 and PC-2, which are required to generate POMC derivatives (26). Furthermore, human endometrial cells express POMC and release one of its end products, β-endorphin (5). It was thus shown for the first time that β-endorphin IRM was released under in vivo conditions from peripheral tissues into the cardiovascular system due to metabolic changes under a state of ischemia. The source of the determined β-endorphin IRM at t0 could be any tissue expressing POMC in the ischemic leg, e.g., skin [dermal endothelial cells (26)], immune cells (33), nerves, skeletal muscles (14), or the vascular system, including vascular endothelial cells (5). Although the functional target of this process may be the same as for POMC release from the pituitary under stress, it is still a matter of speculation. The stimulus of the β-endorphin IRM release might have been the tissue damage by surgery leading to disruption of cell membranes and release of cell constituents; however, just ischemia with all of its effects might have been the reason: lack of oxygen and nutrients or the missing removal of metabolites, pH, or BE shifts.

Although at tc and tdp the β-endorphin IRM concentrations in the reperfusion blood were below the detection limit (Fig. 2), the ACTH concentrations increased from tc to tdp. The time lag (~10 min) between ischemia and ACTH response was more consistent with the known response latency of ACTH release from the pituitary in response to various stressors (18, 31).

This is the first time that the release of N-acetyl-β-endorphin IRM into the cardiovascular system under ischemic conditions such as acidosis, alteration of lactate concentration, or BE has been investigated. Only small levels of N-acetyl-β-endorphin IRM in plasma were measurable in all patients. Thus the melanophore-type enzyme system of POMC-expressing cells in the anterior pituitary lobe and in tissues apart from the pituitary was not activated under acidosis, alteration of lactate concentration, BE, or other perioperative conditions studied here.

Identity of β-Endorphin IRM Released from Ischemic Tissue

In several studies, the magnitude of the exercise-induced increase of peripheral blood β-endorphin levels ranged from 1.5-fold (11) to 7-fold (23). However, it was not only β-endorphin(1-31) but primarily β-endorphin IRM, which was always determined in those studies. To obtain information as to whether in fact β-endorphin(1-31) is released under anaerobic conditions achieved by tourniquet-induced ischemia, we analyzed the reperfusion blood plasma with a highly specific β-endorphin(1-31) assay (12) in addition to the determination of β-endorphin IRM. In reperfusion blood at t0, we observed β-endorphin(1-31) in only a few samples, which contained amounts just above the detection limit. It remains to be clarified in future investigations which kind of β-endorphin derivative was released from tissues apart from the pituitary into the cardiovascular system under anaerobic conditions.

BE: A Major Stimulus of β-Endorphin Release

In the present study, the initial data at tA differ for each patient; therefore, the Spearman’s rank correlation coefficients were calculated for differences between POMC derivative levels in reperfusion blood plasma before tourniquet release and t0 vs. lactate concentration and blood-gas parameters. A significant correlation was found for the alteration of BE with the change of β-endorphin IRM concentrations, whereas no significant correlation was found between the change of lactate concentration, pH, Po2, or PCO2 with the change of β-endorphin IRM concentrations. This pattern of response suggests that the pH drop with ischemia might not be a parameter critical for the release of β-endorphin IRM from ischemic tissue. Therefore, it is the alteration of BE in ECF rather than acidosis that seems to be a major stimulus for β-endorphin release from ischemic tissues apart from the pituitary. However, our findings are consistent with observations reported by Taylor et al. (32) for athletes undergoing incremental or constant exercises.

In the present study, we observed increased levels of BE (from tA to t0) in the reperfusion blood after a period of ischemia (Table 2). The reperfusion blood contained a high PCO2 and a high lactic acid concentration. The increased production of metabolites, such as lactic acid, leads to metabolic acidosis, and the increased production of CO2 leads to respiratory acidosis. Metabolic acidosis leads to shift to the right in Eq. 2; respiratory acidosis induces a shift to the left. In the cardiovascular system of the whole organism, metabolic or respiratory compensation by renal bicarbonate or respiratory CO2 removal occurs. However, because for the tourniquet leg these compensatory mechanisms are entirely
excluded, pH or BE directly related to bicarbonate (HCO₃⁻) concentration are just results of the equilibrium based on the strength of the two shifts.

HCO₃⁻ + H⁺ ⇌ H₂CO₃ ⇌ H₂O + CO₂

(2)

Apparently under the conditions of the tourniquet leg, the “metabolic shift” to the right of Eq. 2 is overruled by the “respiratory shift” to the left, resulting in an increase of bicarbonate concentration and thus a “positive BE.” In fact, increasing levels of actual bicarbonate after tourniquet deflation were determined (Table 2), which were responsible for a positive BE, as may be derived from Eq. 1 (see METHODS).

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REFERENCES


