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IN VITRO BIOSYNTHESIS AND N-ACETYLATION OF ß-ENDORPHIN IN PARS INTERMEDIA OF THE RAT PITUITARY

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SUMMARY: The in vitro biosynthesis of ß-endorphin and its subsequent N-acetylation was studied in the pars intermedia/nervosa (PIN) of the rat pituitary. Freshly isolated PINs were incubated with ^{3}H-tyrosine for various periods of time. Tissue extracts were subjected to double-immunoprecipitation with an antiserum raised against ß-endorphin. The immunoprecipitated compounds of a molecular weight corresponding to that of ß-endorphin were separated from ß-endorphin precursor molecules by gel-chromatography and subjected to chymotryptic cleavage. ^{3}H-tyrosine- or N-acetyl-^{3}H-tyrosine-containing peptide fragments were analysed by thin-layer chromatography. It was found that N-acetylation of ß-endorphin occurred immediately after its formation from its precursor molecules as a modification step closely linked with the post-translational formation of ß-endorphin. Up to 70% of newly synthesized radiolabelled ß-endorphin was found to be N-acetylated during the course of prolonged in vitro incorporation.

ß-Endorphin and adrenocorticotropin (ACTH) have been shown by Mains et al. (1) to be derived from a common precursor molecule (31 K ACTH/ß-endorphin (1), pro-opiocortin (2)). In the pars intermedia of the rat pituitary, the synthesis of pro-opiocortin is followed by its complete processing into ß-endorphin and α-melanocyte-stimulating hormone (α-MSH) (3,4,5). The N-terminal acetyl-group of α-MSH (N-acetyl-ACTH$_{1-13}$-amide) is of importance for the biological (melanophoric) activity of this peptide (6). In contrast, ß-endorphin, which can also undergo N-acetylation on its N-terminus (7,8), is markedly reduced in its activity as an opiate agonist after N-acetylation, as has been demonstrated in a variety of analgesic tests (7,8), in an in vitro opiate receptor binding assay (7,8) and on the guinea pig isolated longitudinal muscle preparation (9).

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The present paper is concerned with an attempt to study the time-course of the N-acetylation of newly synthesized ß-endorphin and the temporal connection of the N-acetylation with the post-translational formation of ß-endorphin from its precursors by use of an in vitro pulse-labelling technique.

MATERIALS AND METHODS

In vitro incorporation of ^{3}H-tyrosine into pituitary lobes

Male Sprague-Dawley rats, weighing 200-220 g, were decapitated, their brains quickly removed and pituitaries divided in situ into the anterior lobes and the posterior lobes with adhering intermediate lobes (PINs).

The freshly dissected PINs were placed into a vessel containing Krebs-Ringer-bicarbonate (KRB) solution and preincubated for 1-2 hrs at 37°C under constant bubbling with carbogen (95% O_2; 5% CO_2) (10). Thereafter, the PINs were transferred into incubation tubes (3 PINs per tube) containing 1 ml KRB, 0.1% bovine serum albumine (BSA), 0.3 mCi tyrosine-L-[ring-3.5-^{3}H] (52.5 Ci/mmole = 1.94 TBq/mmole, NEN, Dreieich, FRG). At various time intervals after incubation (30, 45, 60, 90, 120, 240 min, 8 hrs and 16 hrs) with constant shaking at 37°C in a humidified, carbogen-containing atmosphere, the pituitary lobes were removed and immediately homogenized with 0.5 ml of ice-chilled 5 M acetic acid containing the enzyme inhibitors phenylmethylsulfonylfluoride (PMSF, 0.3 mg/ml) and iodoacetamide (IAA, 0.3 mg/ml) and 5 mg/ml BSA as described by Mains and Eipper (11). After centrifugation at 20.000 g for 10 min and 4°C, the supernatants were lyophilized and reextracted in 0.5 ml 1% (v/v) acetic acid-containing PMSF and IAA (Sigma, Taufkirchen, FRG). After a further centrifugation, the final supernatants were lyophilized and redissolved in 500 µl "buffer D" (0.2 M sodium phosphate, 0.15 M NaCl, 0.01 BSA, 0.1% Triton-X-100, 0.1% gelatin and 0.01% thimerosal, pH 7.4, according to the protocol of Guillemin et al. (12)).

Double-immunoprecipitation

Aliquots of the final extracts were subjected to a double-immunoprecipitation scheme: 20 µl extracts were incubated for 16 hrs together with 5 µl undiluted ß-endorphin antiserum "HO" raised against synthetic human ß-endorphin (see: Results and Discussion), 10 µl buffer E and 25 µl buffer F at 4°C (buffers according to Mains and Eipper (11)).

Thereafter, 2.5 I.U. of goat-anti-rabbit-γ-globulin (Calbiochem, La Jolla, California, USA) in a 300 µl volume of buffer D was added, the incubation continued for 5 hrs and the thus formed immunoprecipitates centrifuged (20.000g/10 min/4°C). Pellets were washed with 500 µl buffer D and recentrifuged. The immuno-complexes were disaggregated by dissolving the final pellets with 200 µl 10% (v/v) acetic acid and incubating for 15 min at 96°C.

Gel-filtration

Disaggregated immunoprecipitates from each pulse period were subjected to gel-filtration Sephadex-G-50 superfine columns (0.9 x 90 cm) and eluted with 10% (v/v) acetic acid at 6°C. 1 ml fractions were collected at a flow rate of 5 ml/h. The column was calibrated with BSA for the void volume;

the elution volumes of porcine ß-LPH ($ß_p$-LPH) (a gift from Dr. L. Gráf, Budapest, Hungary), ß-endorphin and various derivatives of ß-endorphin were estimated by radioimmunoassay using antiserum "HO". Synthetic camel ß-endorphin ($ß_c$-endorphin), synthetic camel N-acetyl-ß-endorphin (N-acetyl-$ß_c$-endorphin), synthetic human ß-endorphin ($ß_h$-endorphin) (all obtained from Peninsula, San Carlos, USA) and $ß_p$-LPH_{61-87} eluted in the same fractions.

Fractions containing radioactive immunoprecipitated material which comigrated with the ß-endorphin standards as a single peak were pooled, lyophilized and subjected to chymotryptic cleavage.

Chymotryptic cleavage

After gel-filtration, the pooled and lyophilized fractions containing radiolabelled immunocomponents of a molecular size approximately equal to that of ß-endorphin were redissolved in 30 µl destilled water and adjusted to pH 8.2 with acetic acid and NH_3. 20 µg of bovine α-chymotrypsin (Sigma, Taufkirchen, FRG) was added at time 0, after either 6 or 20 hrs.

After 30 hrs incubation, 1.3 µg L-tyrosine, 50 µg N-acetyl-tyrosine (Sigma, Taufkirchen, FRG), 50 µg synthetic tyr-gly-gly-phe (a gift from Dr. Fasold, Frankfurt, FRG) and 50 µg N-acetyl-tyr-gly-gly-phe were added to each of the samples, which were than lyophilized. (N-acetyl-tyr-gly-gly-phe was obtained by the following procedure: 600 µg tyr-gly-gly-phe was dissolved in 500 µl aqua dest., 10 µg acetic acid anhydride was added both immediately and after 5 hrs, and the reaction (at 20°C) stopped after 24 hrs by freezing and lyophilizing the sample.)

Thin-layer chromatography of the chymotryptic cleavage products

The samples were redissolved in 30 µl aqua dest., 10 µl were applied to a cellulose chromatography with fluorescence at 254 nm (DC-Plastikfolien, Cellulose F_{254}, Merck, Darmstadt, FRG).

The same standards added to the tubes after chymotryptic cleavage, were chromatographed in parallel. Table 1 shows the relative mobilities to the solvent front of tyrosine, tyr-gly-gly-phe, N-acetyl-tyr and N-acetyl-tyr-gly-gly-phe for 3 different solvent systems which were also used for the endogenous cleavage products. The standards were visualized either by fluorescence extinction (N-acetyl-tyr and N-acetyl-tyr-gly-gly-phe) or by staining with ninhydrin (tyrosine and tyr-gly-gly-phe). For detection of the endogenous radiolabelled cleavage products, the thin-layer plates were cut into 5 mm stripes, dissolved in 15 ml scintillation fluid (Aqualuma plus, Baker, Groß-Gerau, FRG), shaken for 16 hrs and counted for radioactivity.

TABLE I: Thin-layer chromatography of synthetic fragments, equivalent to the N-terminal chymotryptic cleavage products of ß-endorphin

running buffer standards	butanol:acetic-acid:water (4:1:1)	methanol:pyridine:water (20:1:5)	chloroform:methanol:NH_3 (20:20:6)
	relative mobilities to the solvent front		
tyrosine	0.32	0.43	0.41
tyr-gly-gly-phe	0.56	0.61	0.53
N-acetyl-tyr	0.79	0.79	0.54
N-acetyl-tyr-gly-gly-phe	0.80	0.81	0.66

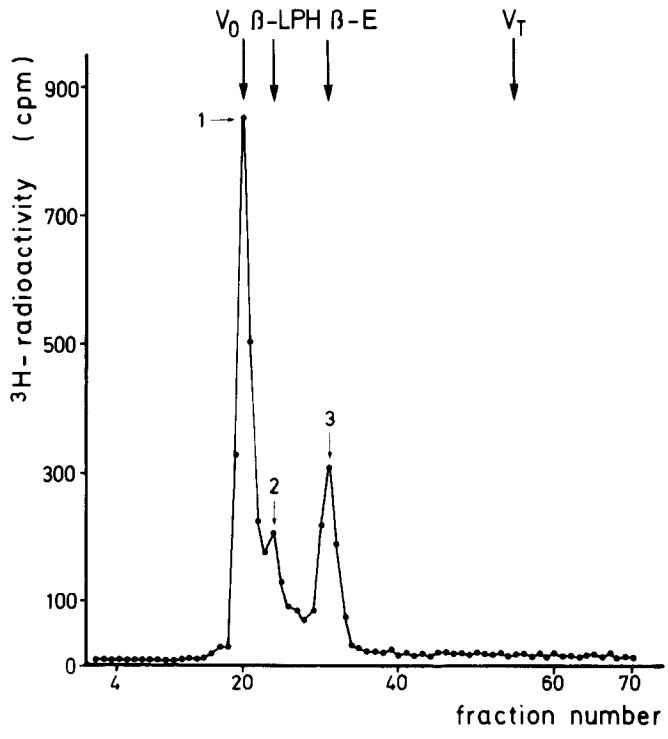

FIGURE 1: Gel-filtration of ^{3}H-tyrosine-labelled peptides in rat intermediate/posterior lobes on Sephadex-G-50. The pituitary lobes were incubated in vitro with ^{3}H-tyrosine for 2 hours. Prior to gel-filtration, the radiolabelled peptides which bind to ß-endorphin antiserum "HO" were isolated by double-immunoprecipitation as described in Methods. Arrows indicate the void volume (=V_o), the total volume (=V_t) and the elution fractions of $ß_p$-lipotropin (=ß-LPH) and synthetic $ß_c$-endorphin which coelutes with synthetic N-acetyl-$ß_c$-endorphin and $ß_p$-LPH_{61-87} (all together: ß-E). All endogenous ^{3}H-tyrosine-labelled peaks could be completely eliminated by addition of 5 µg unlabelled $ß_c$-endorphin to the aliquots of extract applied to double-immunoprecipitation.

RESULTS AND DISCUSSION

Isolated intermediate/posterior lobes of rat pituitaries were incubated together with ^{3}H-tyrosine for various periods of time. After subjecting the tissue extracts to double-immunoprecipitation, using an antiserum raised against ß-endorphin, the redissolved immunoprecipitates were subjected to gel-filtration on a Sephadex-G-50 column.

Figure 1 depicts the immunoprecipitated compounds, separated by a Sephadex-G-50 column, contained in the extract obtained from a 2 h pulse with ^{3}H-tyrosine. Peak 1) in the void volume probably corresponds to pro-opiocortin, peak 2) coelutes with $ß_p$-lipotropin , and peak 3)

with synthetic β_c-endorphin, N-acetyl-β_c-endorphin and β_p-LPH_{61-87} (C'-fragment). Therefore, the endogenous radiolabelled "ß-endorphin-peak" (peak 3, Fig. 1) may not only comprise ß-endorphin (ß-LPH_{61-91}), but also closely related derivatives, such as N-acetyl-ß-endorphin, C'-fragment and its N-acetylated counterpart, since they have been demonstrated to also occur in the rat pituitary (8) and are recognized by our antiserum "HO".

The antiserum "HO" exhibits an identical avidity for synthetic β_c-endorphin and N-acetyl-β_c-endorphin which possess the same structures as their counterparts in the rat (13). In view of the non-availability of rat ß-LPH_{61-87} and N-acetyl-ß-LPH_{61-87}, we are unable to provide any precise information concerning the affinity of the antiserum for these peptides. Since β_p-LPH_{61-87} is easily recognized by the antiserum, (only 2-3-fold less than β_c-endorphin), it is likely, however, that the antiserum might also display a high avidity for the structurally closely related rat ß-LPH_{61-87} and its N-acetylated derivative. However, even a saturating 3-fold increase in the concentration of the ß-endorphin antiserum used for the double-immunoprecipitation produces no alteration in the amount of N-acetylation of ß-endorphin observed. Thus, at a high (supersaturating) antiserum concentration virtually all N-acetylated or non-N-acetylated rat ß-endorphin fragments should be quantitatively immunoprecipitated (for further characterization of the antisersum "HO" see (14)).

In an effort to determine the proportion of "ß-endorphin" which underwent N-acetylation ("ß-endorphin", consisting here of ß-LPH_{61-91} and its fragment ß-LPH_{61-87}), the fractions of the "ß-endorphin peak" obtained from the Sephadex-G-50 column for each pulse period were subjected to enzymatic degradation by α-chymotrypsin and the products analysed by thin-layer chromatography. Four ^{3}H-tyrosine-labelled chymotryptic cleavage products (^{3}H-tyrosine, N-acetyl-^{3}H-tyr, ^{3}H-tyr-gly-gly-phe and N-acetyl-^{3}H-tyr-gly-gly-phe), each derived from the N-terminus of ß-endorphin, where

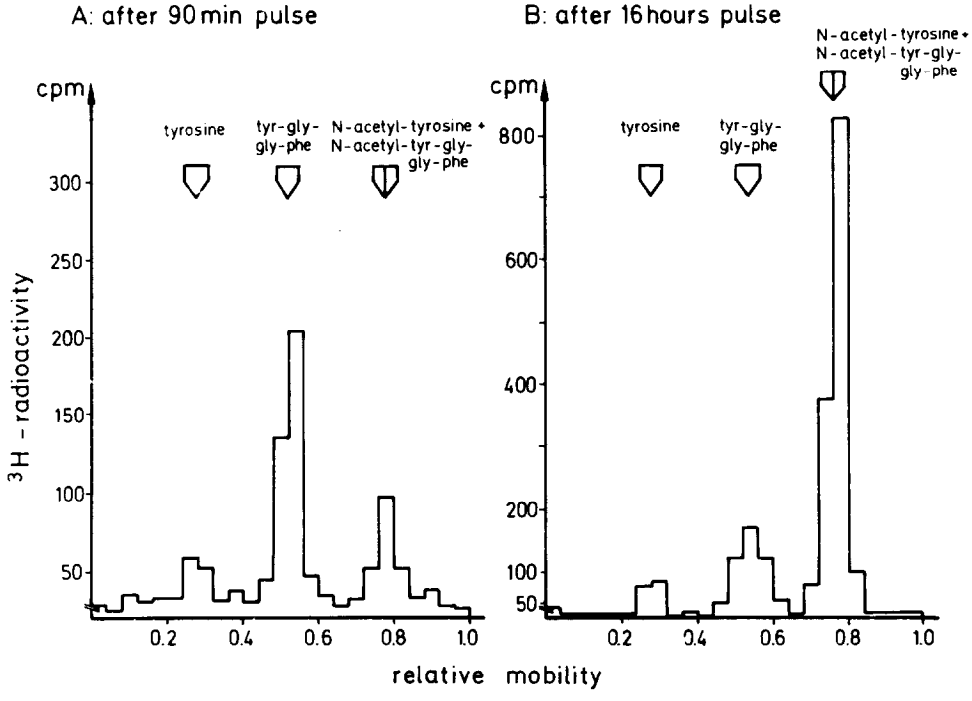

FIGURE 2: Thin-layer chromatography of chymotryptic cleavage fragments of ^{3}H-tyrosine-labelled ß-endorphin-like peptides. Isolated intermediate/posterior lobes were incubated with ^{3}H-tyrosine for 90 min (A) or 16 hrs (B). Thereafter, the radiolabelled peptides in the tissue extracts which bind to ß-endorphin antiserum "HO" were isolated by double-immunoprecipitation; the peptides with a molecular size approximately equal to that of ß-endorphin were purified by gel-filtration (see also Fig. 1) and subjected to chymotryptic cleavage. The final chymotryptic products were spotted on cellulose thin-layer sheets and developed in a solvent containing butanol:acetic acid:water (4:1:1). The thin-layer plates were cut into 5 mm stripes, dissolved in 15 ml scintillation fluid, shaken for 16 hrs and counted for radioactivity. Arrows indicate the relative mobility of the synthetic fragments identical with the N-terminal chymotryptic cleavage products of ß-endorphin (for details see Methods).

the only tyrosine residue in this peptide is located, could be identified by virtue of their comigration with their respective unlabelled standards in 3 different solvent systems (see: Methods). Figure 2 shows the migration profile of the radiolabelled chymotryptic cleavage products of immunoprecipitated ß-endorphin-like peptides after incubation of isolated PINs with ^{3}H-tyrosine for 90 min (3A) or 16 hrs (3B), obtained from cellulose thin-layer chromatography running in butanol (4): acetic acid (1): water (1).

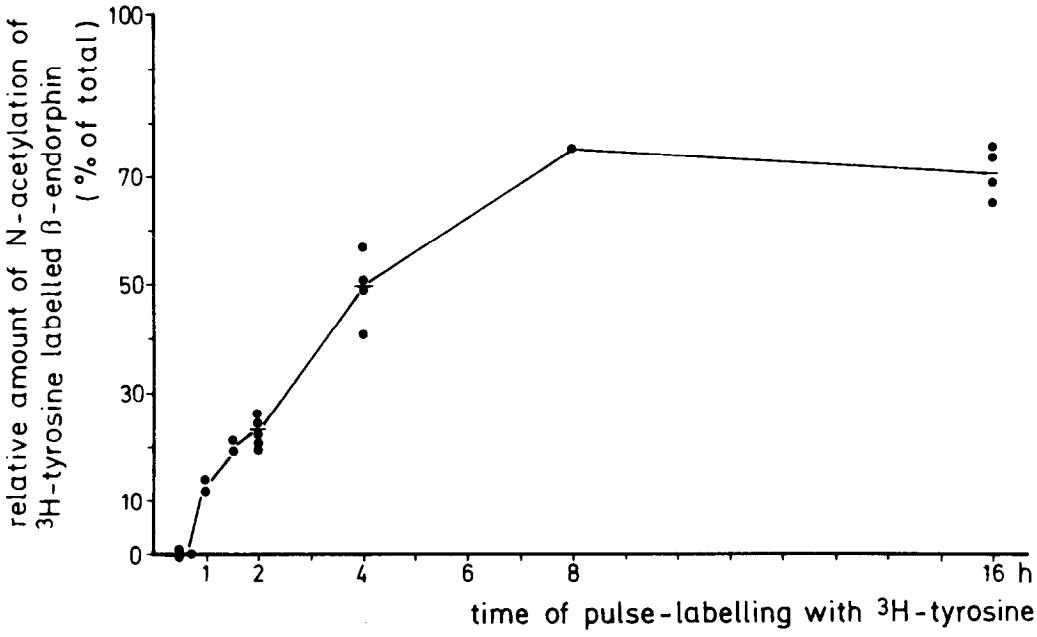

FIGURE 3: Time-course of the N-acetylation of ^{3}H-tyrosine-labelled ß-endorphin-like peptides as estimated from the relative amounts of the N-acetylated and non-N-acetylated ^{3}H-tyrosine-labelled chymotryptic cleavage fragments obtained by the procedure described in legend of figure 2.

It can be seen that after 90 min incubation chymotryptic cleavage of the ^{3}H-tyrosine-labelled ß-endorphin produces predominantly non-N-acetylated fragments (^{3}H-tyrosine + ^{3}H-tyr-gly-gly-phe > N-acetyl-^{3}H-tyr + N-acetyl-^{3}H-tyr-gly-gly-phe), whereas after 16 hrs incubation, the majority of the chymotryptic cleavage products are N-acetylated.

Figure 3 illustrates the time-course of the N-acetylation of ^{3}H-tyrosine-labelled ß-endorphin over a period of 16 hrs, as estimated from the relative amounts of the N-acetylated and non-N-acetylated ^{3}H-tyrosine-labelled chymotryptic products obtained. It may be seen that there is a steady increase in the quantity of N-acetylated ^{3}H-tyrosine-labelled ß-endorphin versus total ^{3}H-tyrosine-labelled ß-endorphin observed between 1 h and 4 hrs of incubation. A maximal degree of N-acetylation (70%) was present at 8 hrs with no further increase apparent by 16 hours.

An about 45 min pulse time with tritiated amino acids was necessary for the appearance of radiolabelled ß-endorphin - the time required for the synthesis of pro-opiocortin and its processing into ß-lipotropin and subsequently into ß-endorphin (data not shown, see (14)). The fact that N-acetylated ß-endorphin could be demonstrated as early as after a 60 min pulse time (already ∿12% of the total radiolabelled ß-endorphin was N-acetylated), is strongly indicative, that the N-acetylation is intimately linked with the post-translational formation of ß-endorphin.

In the present study, the time-course of the N-acetylation of not only ß-endorphin but also of ß-endorphin fragments possessing a similar molecular weight, such as the C'-fragment (= ß-LPH_{61-87}), was concurrently evaluated. It may be that the time-course and the extent of the N-acetylation of ß-endorphin differs from that of the C'-fragment. Moreover, formation of the C'-fragment from ß-endorphin might also be regarded (as with N-acetylation) as another post-translational modification leading to products exhibiting less opiate-like activity than ß-endorphin in analgesic tests (7).

However, N-acetylation seems to be the basic mechanism for inactivation of ß-endorphin, since in the guinea pig isolated longitudinal muscle preparation C'-fragment and ß-endorphin are almost equipotent, whereas N-acetylated ß-endorphin exhibits no detectable opiate activity (9).

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