Kyotorphin Suppresses Proliferation and Ca^{2+} Signaling in Brown Preadipocytes

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A positive correlation was revealed between stimulation of protein and DNA synthesis in preadipocytes by norepinephrine or neokyotorphin and intracellular Ca^{2+} concentration in these cells. Kyotorphin abolished the stimulatory effect of norepinephrine on proliferation of cultured cells and cold-induced [^{3}H]-thymidine incorporation into DNA of mouse brown adipose tissue *in vivo*. These changes correlated with peptide-induced suppression of slow calcium signaling in preadipocytes.

Key Words: *brown adipocytes; preadipocyte proliferation; calcium signaling; kyotorphin; neokyotorphin*

Noncontractile thermogenesis plays an important role in the survival of small animals in cold and is realized via activity of brown adipose tissue (BAT) [2]. Hyperplasia and hypertrophy of BAT under cold conditions are mediated by activation of the sympathetic nervous system [6,8]. The release of norepinephrine (NE) into BAT not only stimulates proliferation of brown adipocytes, but also prevents apoptosis in these cells [9]. The release of NE is suppressed in animals maintained at 22-25°C, which results in apoptosis and involution of BAT.

Hyperphagia in hibernators is accompanied by accumulation of brown and white fat. Activation of the sympathetic nervous system and hyperplasia of BAT in non-hibernating mice and rats prevent accumulation of white fat. However, the formation of BAT is not necessarily associated with activation of the sympathetic nervous system. The formation of BAT in newborns of some species starts during the prenatal period. In hibernating animals (*e.g.*, gophers) the formation and involution of BAT are characterized by seasonal variations. Temperature regulation plays a secondary role in these animals [8]. Hyperplasia of BAT is probably regulated not only by the adrenergic system, but also by other endogenous compounds. The search for endogenous compounds regulating hibernation resulted in isolation of several tens of peptides [1], *e.g.* neuropeptides kyotorphin (YR) and neokyotorphin (TSKYR). These peptides exhibit thermoregulatory activity [1]. Here we studied regulatory properties of these peptides.

MATERIALS AND METHODS

Brown preadipocytes were isolated from BAT of 3-4-week-old mice by the collagenase method. The cells were purified by centrifugation [5] and inoculated into 6-well plates with DMEM medium containing 10% fetal bovine serum and insulin for 3 days. Then the medium was replaced with a serum-free medium and experiments were performed after 24 h [4]. NE (1 μM) and TSKYR (1-100 nM) were added to wells. The cells were sampled after 16-20 h. The concentrations of DNA and protein were measured using Hoechst-33258 and Coomassi Blue G-250, respectively [5]. The concentrations of protein and DNA in control samples of each series were taken as 100%. The estimated values were

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standardized. The mean value was calculated from the results of 4-5 experiments. Each additive was tested in 2-4 wells. In various experiments the 100% values corresponded to 2-5 μg DNA and 30-70 μg total protein. Changes in intracellular Ca^{2+} concentration in freshly isolated preadipocytes was recorded using a Fura-2AM probe [4,7]. Cytoplasmic Ca^{2+} concentration was measured 20 min after individual or combined treatment with 3 μM NE and 3 μM TSKYR. The mean value was calculated from the results of 4 experiments.

The tests were performed as described elsewhere [10]. Instead of 24-h cold exposure, the animals were subjected to intermittent short-term cold exposure [11]. They were divided into 6 groups (n=4-5). Two groups of mice were maintained at 21-23°C. The animals of 4 groups were placed in a cold room for 5 h (4°C, 9.00-14.00). The test solutions (0.3 ml) were injected at 9.00 and 11.30. The mice received physiological saline (1 group in cold, 1 group in heat); 0.03, 0.10, and 0.30 mM solution of YR in sterile physiological saline (3 groups in cold); and 0.1 mM solution of YR (1 group in heat). The treatment was performed for 5 days. [^{3}H]-Thymidine in a dose of 16 μCi was injected on day 5 (20.00). The mice were killed after 4 h. The interscapular BAT was removed, homogenized in physiological saline and 20 mM phosphate buffered saline (pH 7.2), and frozen at -30°C. DNA concentration and [^{3}H]-thymidine incorporation in samples were studied on the next day [5,10]. Here we presented the results of 3 experiments.

RESULTS

TSKYR and NE produced an additive stimulatory effect on proliferation of brown fat cells (Fig. 1). A correlation was revealed between the effect of TSKYR and/or NE on preadipocyte proliferation and increase in intracellular Ca^{2+} concentration (Fig. 1). We studied the antagonistic effect of YR on NE-induced growth of preadipocytes. YR in doses of 10-100 nM blocked the effect of 1 μM NE. However, in other doses the peptide was ineffective (Fig. 2, *a*).

The negative effect of YR on NE-induced synthesis of DNA (Fig. 2, *a*) in cultured cells and inhibition of the Ca^{2+} response in freshly isolated cells to NE were observed only in a narrow range of peptide concentrations (Fig. 2, *b*). The use of NE in various concentrations allowed us to estimate the most effective concentration of YR (Fig. 2, *a*, *b*). However, the YR/NE ratio remained practically unchanged in various series (1:10).

The negative effect of YR on DNA synthesis in brown adipocytes was studied *in vivo* to support the hypothesis that the influence of this peptide on cultured cells is not just a laboratory phenomenon. Proliferation of preadipocytes in mouse BAT was induced with a natural stimulator, cold exposure (instead of NE). The animals were subjected to intermittent short-term cold exposure. YR was administered at the moment of cold exposure to suppress NA-induced stimulation of DNA synthesis in BAT. Injection of YR in a certain concentration was followed by suppression of [^{3}H]-thymidine incorporation (Fig. 2, *c*). The peptide was effective only in a narrow range of concentrations. Treatment with the peptide in a 3-fold higher or lower concentration did not suppress DNA synthesis in tissue. The concentrations of YR in *in vivo* experiments were 1.0-1.5 μM.

A significant correlation was revealed between the mitogenic effect of peptides or suppression of mitogenesis in cultured preadipocytes and change in slow calcium signaling in isolated cells. The data indicate that calcium ions play a major role in this regulation. The mechanism for adrenergic activation of slow calcium signaling in young brown fat cells was described previously [7]. However, the mechanism of peptide regulation of reduced calcium signaling remains unknown.

We conclude that peripheral tissues have receptors for these neuropeptides. Our results are consistent with published data that YR and TSKYR regulate proliferation of linear and transformed cells [3]. Both peptides similarly stimulate Ca^{2+} signaling

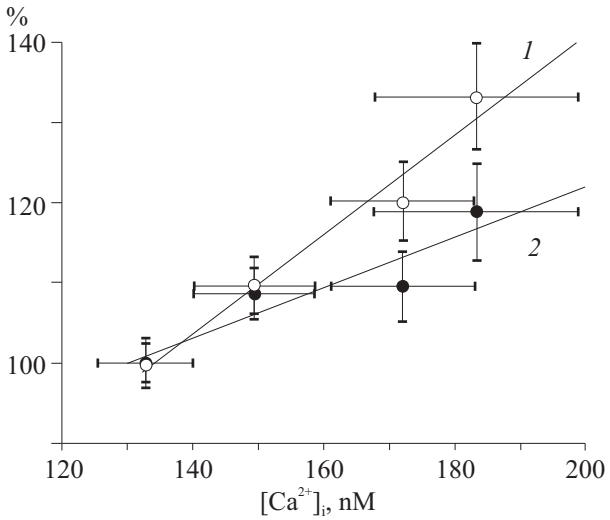

Fig. 1. Correlation between the effects of norepinephrine (NE) and neokyotorphin (TSKYR) on protein and DNA synthesis in proliferating brown adipocytes (days 3-4 of culturing) and intracellular Ca^{2+} concentration in freshly isolated preadipocytes. Correlation coefficients for changes in the concentration of DNA and protein are 0.98 (*1*) and 0.93 (*2*), respectively. Paired symbols correspond to the additives (from left to right): control, NE, TSKYR, and TSKYR+NE. Measurement of protein and DNA: n=(4-5)×(2-4 wells). Measurement of intracellular Ca^{2+} concentration, n=4.

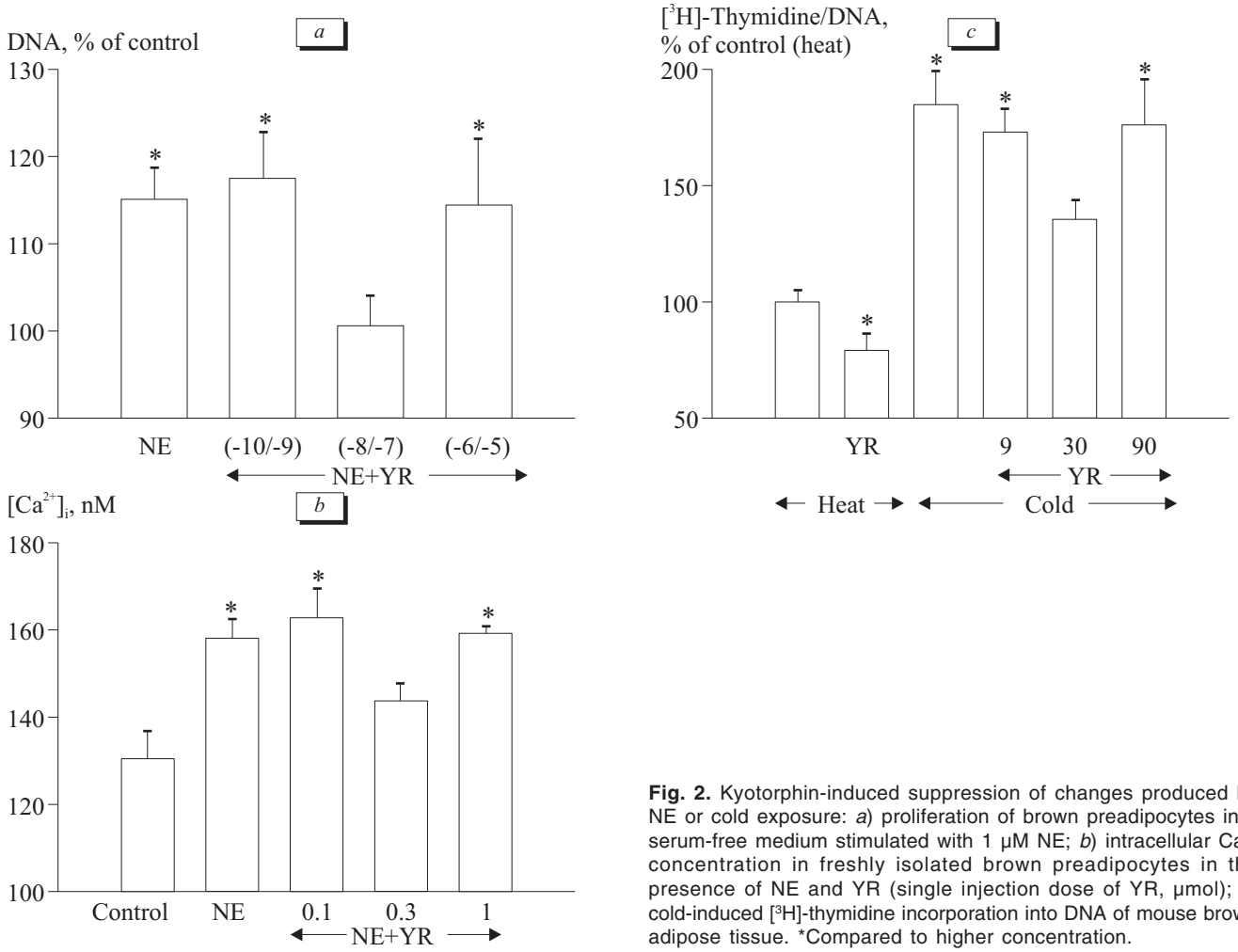

Fig. 2. Kyotorphin-induced suppression of changes produced by NE or cold exposure: *a*) proliferation of brown preadipocytes in a serum-free medium stimulated with 1 μM NE; *b*) intracellular Ca^{2+} concentration in freshly isolated brown preadipocytes in the presence of NE and YR (single injection dose of YR, μmol); *c*) cold-induced [^{3}H]-thymidine incorporation into DNA of mouse brown adipose tissue. *Compared to higher concentration.

[4], but have different effects on proliferation of brown adipocytes. Hyperplasia of BAT in hibernating animals is probably regulated by not only noradrenergic, but also peptidergic system.

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