INSULIN RECEPTORS IN MOUSE BRAIN: REVERSIBILITY OF AGE-RELATED IMPAIRMENTS BY A THYMIC EXTRACT

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ABSTRACT

Recently, we have shown that insulin receptors (InsRs) in the brain undergo impairments with aging. Interestingly, age-related alterations of brain InsRs, are not irreparable as thymus grafts are able to recover them. With the present study we verified the possibility that an aqueous extract from calf thymus (TME) can mimic the restoring action of age-related impairments induced by thymus graft. InsR characteristics were assayed in a group of 25 months old BALB/c-nu mice treated with TME: 2µg/g body weight every third day, for total five subcutaneous injections. The last dose was injected the day before animals were killed. Other two groups of young (4 months) and old (25 months) mice received saline solution with the same schedule. A two-sites model analysis of receptor data confirms the age-dependent decrease of InsR number and k_d previously observed in the high affinity population. Furthermore, a statistically significant recovery of number impairment is shown in TME-treated animals. On the contrary, the characteristics of the low affinity receptor subset show no statistically significant differences among the three animal models studied. TME induced recovery of the age-related changes found in brain InsRs, together with previously observed regulatory action of the same thymic extract on the adrenergic system, suggest that thymic gland does not necessarily have to mutually interact with other controlling systems for maintaining or recovering homeostasis of the complex neuroendocrine network during development and aging.

INTRODUCTION

Central insulin and insulin receptors (InsRs) are widely distributed throughout the CNS (1-5), where insulin mainly fulfills functions different from the classical one of glucose metabolism control. Examples of central hormone actions are regulation of development and growth of CNS (1, 2), neuromodulation of monoaminergic systems (6), regulation of food intake and body weight (7, 8), and regulation of peripheral glucose homeostasis (9, 10). These functions are mediated through specific brain InsRs, unique to neuronal cells, which are indistinguish-

able from peripheral ones regarding binding kinetics (11, 12); however, they differ in some biochemical and physiological properties. For example, neuronal InsRs are smaller in size (13-15), do not undergo neither internalization nor down-regulation, but instead are up-regulated by their own hormone (4, 16-18).

Recently, studies from our laboratory on the low and high affinity InsR binding sites in mouse brain have shown that these receptors, as it happens for other receptor systems, exhibit altered functions with aging. In particular, receptor number of the high affinity subset is significantly decreased in old mice (19). Particularly intriguing is the finding that such alterations are not permanent, as they can be restored by a thymus graft (20). In fact, a neonatal thymus, grafted into old recipients, is able to correct the impaired number values of brain InsRs to the levels of young counterparts. It is worth noting that the restorative action of the thymus was previously observed also on age-related modifications of several biological functions at level of different tissues. Examples are represented by DNA synthesis in hepatocytes (21), blood levels of insulin and thyroid hormones (22), and β -adrenergic functions in brain cortex and submandibular glands (23-26).

Thus, regarding CNS functions of insulin, it can be hypothesized that age-dependent modifications observed on brain InsRs contribute to alterations of several functions at different physio-pathological levels, such as increase of fat body mass with aging or late onset of glucose intolerance. The same changes can also be involved in the pathogenesis of some relevant age-related diseases, such as senile dementia of Alzheimer type and Werner syndrome (27-29). The possibility of correcting some age-dependent alterations by thymus graft raises the question of whether a treatment with a thymic extract can also be effective in restoring age-related modifications of brain InsRs.

In addition to gained insight on the involved mechanisms, the restorative action of the thymus may open a research line on possible future applicability to humans. Among the many available factors, we started using TME, an aqueous extract from calf thymus, which is one of the few extracts prepared on the basis of nonimmunological tests (30, 31); therefore, it is particularly useful in our study of endocrine actions of the

thymus. Furthermore, TME is crude enough to assure the presence of several active thymic molecules and, what is more, it has been already demonstrated to have an effect on some age-related alterations of nonimmunologic parameters previously shown recoverable by thymus graft (32, 33).

On these bases, we performed an experiment to verify the capability of TME to correct the modifications observed in brain InsRs from old mice. Thus, the characteristics of high and low affinity binding sites were studied in the brain of old BALB/c-nu mice treated with TME and compared with those of young and old control mice.

MATERIALS AND METHODS

Chemicals

[¹²⁵I]-(TyrA14)-monoiodinated human insulin (2000 Ci/mmol) was obtained from Amersham. Unlabelled porcine insulin (24 I.U./mg protein), bacitracin (50000 units/g), bovine serum albumin (BSA) fraction V, Tris-HCl were purchased from Sigma and HEPES (buffer solution 1 M) was obtained from Gibco. TME was a kind gift of Dr. D. Amici, the preparation of which is described below. All other chemicals were reagent grade.

Animals

Animals were BALB/c-nu from our own colony, the same strain previously used in studies on aging. The term "nu" refers to the recessive mutation of nudity introduced in inbred BALB/c crossing them with nude mutants. It is worth noting that BALB/c-nu mice are a long living strain, with a median life span of about 28 months. These animals are not characterized by immunodeficiency despite the presence of the "nu" mutation; on the contrary, the thymus is still present at late age, weighting about 10 mg at the age of 28 months vs about 27 mg in 4 months old mice. Three animal groups were considered: 8 old mice (25 months) treated with TME; 5 young (4 months) and 6 old (25 months) mice as controls.

Mice treated with TME (2µg/g body weight) were injected subcutaneously every third day for total five injections. The last dose was administered the day before animals were suppressed for binding experiments. Young and old control mice received saline solution with the same schedule used for TME-treated mice. Animals were killed by cervical dislocation at the same hour in the morning, then brains were quickly removed and immediately used for binding assay.

Thymic extract preparation

The aqueous extract TME was prepared according to the method previously described (30) by skipping the ultrafiltration step (32, 33). Briefly, a calf thymus was carefully sectioned and the medullar portion was removed by scraping and homogenized in 2.5 volumes of distilled water. After addition of acetic acid (17.5 mmol/l final concentration) and two volumes of methanol to the homogenate, the preparation was left to stand 24 hrs. The precipitate was removed by centrifugation (20000 x g, 15 min) and the methanol was evaporated in vacuum

at 30°C. Subsequently, the aqueous extract, concentrated three times following evaporation process, was ultracentrifuged (80000 x g), and the supernatant was dialyzed against distilled water for 5 hrs. The aqueous extract was then assayed for protein content according to Lowry et al. (34) and lyophilized.

Membrane preparation

Brains were minced and washed three times in ice-cold buffer containing 0.25 mol/l sucrose, 10 mmol/l Tris-HCl (pH 7.5) and 10 mmol/l EDTA. Each brain was then homogenized in Tris-HCl buffer (50 mmol/l) containing 10 mmol/l MgCl₂. The homogenates were strained through gauze and centrifuged at 15000 x g for 15 min, and membranes were resuspended in the incubation buffer without BSA at a protein concentration of about 1 mg/ml. An aliquot from each membrane preparation was used for protein content determination by the method of Lowry et al. (34) with BSA as the standard.

Binding Assay

Insulin binding to brain membranes was performed incubating 200 µl of membrane preparation in the incubation buffer in a final volume of 1 ml at 4°C overnight in 1.8 ml polyethylene microfuge tubes (Beckman). The incubation buffer was composed of 100 mmol/l HEPES (pH 7.8), 120 mmol/l NaCl, 1.5 mmol/l Na acetate, 2.5 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.0 mmol/l EDTA, 10 mmol/l glucose and 0.1% BSA. Bacitracin (100 U/ml) was added to reduce ¹²⁵I-insulin degradation (35). Insulin binding competition experiments were set up in the presence of 1.02x10² pmol/l of 2000 Ci/mmol ¹²⁵I-insulin and unlabelled porcine insulin over a concentration range of 0-8.7x10⁶ pmol/l. Each experimental competition curve was performed in duplicate. At the end of incubation, assay mixtures were centrifuged at 12000 rpm for 1 min in a refrigerated Eppendorf microfuge, supernatants were decanted, and the pellets were washed with 1 ml of cold HEPES buffer, then counted. All the operations were carried out at 2-4°C. Examples of experimental binding curves from single young, old and old TME-treated mice are reported in Figure 1.

Data analysis

Determination of numbers and affinities of the high and low affinity binding sites were obtained analyzing experimental data by the two-sites model of the computer program Ligand (36, 37). Results are expressed as mean ± SEM of values from each animal group. Receptor numbers are referred to protein content of each membrane preparation. Statistical significance of the parameter differences among the three animal models was obtained by variance analysis (one-way ANOVA test) followed by Student-Newman-Keuls test (significance levels: p<0.05) using the SPSS program.

RESULTS

Analysis of experimental data confirms the age-dependent impairment of receptor characteristics previously

Figure 1: Typical competition binding curves derived from single animals. Absolute insulin binding on brain receptors from young ($\circ$), old ($\square$), and TME-treated old (∇) mice are reported after non-specific binding subtraction. In the examples given in the figure non-specific binding has been calculated as that observed at the highest cold insulin concentration. Data reported in the Figures 2 and 3 were obtained using the program Ligand, which gives direct estimate of non-specific binding by fitting the whole curve obtained from unprocessed data.

observed in the high affinity receptor subset. Figure 2 shows the high affinity receptor number observed in the three animal models. Old mice show a statistically significant decrease of number when compared with young controls. TME-treated old animals show a statistically significant recovery of this alteration. It is also worth noting that receptor number values from young and TME-treated old mice do not show any statistically significant difference, that is, there is a complete recovery. Light differences in protein content are sometimes observed between young and old mice; however, they cannot influence the restorative action exerted by TME as it occurs in the same group of old animals also used as old controls. A similar trend can be observed in Figure 3, which reports data on the k_d of high affinity binding sites. Old animals show a statistically significant decrease of k_d , which, however, only shows a non-statistically significant increasing trend in TME-treated mice.

The low affinity insulin binding sites do not show any statistically significant difference for both number and affinity among the three animal models studied. As observed in previous experimental results, the high degree of interindividual variability found in this population could mask the presence of eventual modifications. At any rate, results on the low affinity insulin subset are hardly interpretable. In fact, the low affinity binding sites can also include IGF-I receptors as well as Ins/IGF-I hybrid receptors which bind insulin with low affinity (38, 39).

Table 1 provides detailed data on the characteristics of both high and low affinity binding sites and the statistical significance of the differences among the three animal models for each receptor characteristic.

Table 1: Effect of TME on age-related modifications of InsRs in mouse brain

	High affinity		Low affinity	
	R (fmol/mg)	k_d (nmol/l)	R (fmol/mg)	k_d (nmol/l)
Young	96.83 $\pm$ 7.45	0.38 $\pm$ 0.04	6629 $\pm$ 1549	0.77 $\pm$ 0.14
Old	59.85 $\pm$ 2.82	0.23 $\pm$ 0.01	4167 $\pm$ 1463	0.42 $\pm$ 0.15
TME-Old	87.82 $\pm$ 5.57	0.30 $\pm$ 0.02	5105 $\pm$ 1847	0.48 $\pm$ 0.14
p (Anova)	0.001	0.003	0.622	0.262

Values are mean $\pm$ SEM. R: receptor number; k_d : equilibrium dissociation constant. Square parentheses show the couples of values whose differences are statistically significant (S-N-K test, $p < 0.05$).

Figure 2: Number of high affinity insulin binding sites in the brain of 5 young ($\square$), 6 old ($\boxtimes$) and 8 TME-treated old ($\boxdot$) BALB/c-nu mice. Results are represented as mean (columns) $\pm$ SEM (bars). TME is able to restore up to the young value the statistically significant decrease of this parameter observed in old animals. Results of statistical analysis are reported in Table 1.

Figure 3: Dissociation constant, k_d , of high affinity insulin binding sites in the brain of 5 young ($\square$), 6 old ($\boxtimes$) and 8 TME-treated old ($\boxdot$) BALB/c-nu mice. Columns and bars represent mean and SEM values, respectively. Old animals show a statistically significant decrease of k_d . TME only induces an increasing trend in k_d , which, however, is not statistically significant. Results of statistical analysis are reported in Table 1.

DISCUSSION

Central insulin and InsRs play an important role in the regulation of basal physiological processes. Knowledge of the mechanisms regulating central insulin action could be crucial in understanding disorders of satiety, such as anorexia nervosa and obesity, and disorders of abnormal glucose regulation, such as adult-onset diabetes mellitus (12). Since glucoregulatory abnormalities

have also been found in diseases, such as senile dementia of Alzheimer type (27, 28, 40) and the premature aging disorder Werner syndrome (29, 41, 42), impairments of central insulin action on glucoregulation could play a role in the etiology and pathogenesis of these diseases. The above mentioned abnormalities are often observed with advancing age, suggesting a role of brain insulin and InsRs in relevant aging processes. In support of this possibility, we previously observed that central InsR characteristics undergo alterations with advancing age. In particular, number and k_d of the high affinity receptor subset show a statistically significant decline in old mice when compared with young controls (12, 19). These data on brain InsRs in aging are among a few available in the literature. In rats data on age-related changes are reported only up to adult age (43, 44) except one study reporting a decrease of brain InsRs only for olfactory bulbs (45). To our knowledge, our data are the first reported in mice. In a recent study in humans, central InsR changes have been observed in autaptic brains of aged individuals (40). Thus, this area of research is likely to expand.

Another line of research that has probably received less attention than due is the role played by endocrine thymus on processes related to aging (46-48) despite numerous studies demonstrate that the thymus regulates two main neuro-endocrine circuits: the hypothalamic-pituitary-gonadal axis and the hypothalamic-pituitary-adrenal axis (49-52). On the other hand, many hormones and neurotransmitters are known to influence development and activity of the thymic gland (53, 54). On these bases, it is not surprising that thymus can control nonimmunological functions in different systems. It also remains quite intriguing the ability of the thymus to restore impaired functions. Our previous studies, in fact, demonstrate that the thymus exerts a regulatory action on some β -adrenergic functions through the mediation of β -adrenoceptor regulation (22, 23). β -adrenergic responsiveness and receptor characteristics, found altered in both young athymic nude and old normal mice, can be restored after grafting a neonatal thymus (22, 23, 47). Thus, thymic gland is needed for both development and maintenance of physiological functions of the β -adrenergic system, but it is also able to recover physiologically relevant age-related alterations. The regulatory influence of thymus has been observed on different tissues, such as submandibular glands, brain cortex and liver, and on different neuronal systems, such as α - and β -adrenergic ones (21, 23, 55-58). The corrective action of the thymus, in addition, does not seem to act in a nonspecific stimulatory way. As a matter of fact, a neonatal thymus grafted into old recipients only exerts a corrective effect just on β_1 -adrenoceptors, the receptor subtype found altered in old animals, leaving unaffected β_2 -adrenoceptors, the subtype not modified by age (25, 59). As mentioned above, the corrective action of the thymus has recently been observed also on aging impairment of brain InsRs (20).

Results from the present study demonstrate that TME can mimic the restorative action of thymus graft on InsRs from whole brain. In fact, TME, administered as described, significantly recovers the age-dependent impairments of brain InsRs. The possibility of recovering alterations of brain InsRs by interventions that are more readily available than thymus graft, such as treatments with thymic extracts or factors, could introduce a new line of research on therapeutical approaches for humans able to prevent or ameliorate the above-mentioned physio-pathologic disorders linked to age-related modifications of central InsRs. In favor of the above-mentioned possibility is also the evidence of the existence of biologically active thymic molecules (60) suggesting that they can mediate some of the multiple inward and outward communication routes possessed by the thymus, though it cannot be excluded that factors from other sources have a similar action. Further support comes from previous experiments demonstrating that TME is able to restore, at least partially, the altered isoproterenol-stimulated DNA synthesis in submandibular glands of old mice (32). Therefore, the question of whether the restorative action of the gland needs mutual interrelationships with the other control systems or, alternatively, even some thymic factors can be effective, seems bending towards the second hypothesis.

A joint analysis of present and previous results also suggests the existence of some common mechanisms involved in the age-related changes and the restorative action exerted by thymus or thymic factors on different targets. Central insulin acts as a neuromodulator of central adrenergic system through α_1 - and/or α_2 -adrenoceptor regulation (61-64). The presence of InsRs on adrenergic terminals suggests that insulin neuromodulatory action is mediated by its central receptors (65). Another important interrelationship between insulin and catecholaminergic system rests in the existence of a feedback loop, localized in the hypothalamus, through which central insulin controls peripheral glucose homeostasis involving adrenergic system in signal relaying (66). The link between insulin and catecholaminergic systems may also assume a remarkable role as far as aging processes are concerned. In fact, in addition to the age-related modifications of brain InsRs (12, 19), it is well established that adrenergic responsiveness is altered during aging, at least partially due to decreased receptor number in both peripheral and central tissues (58, 59, 67). Thus, the restorative action induced by thymus on the age-dependent alterations of brain InsRs and adrenoceptors as well as their interaction during development and aging suggest that the mechanisms regulating age-dependent modifications of these two receptor systems are tightly co-related, or even common, and can represent one of the targets of the restorative action of both thymus and thymic factors.

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