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Effects of Age on Elements of Insulin-Signaling Pathway in Central Nervous System of Rats

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Insulin resistance is known to play a pivotal role in type 2 diabetes. Senile individuals, besides being prone to insulin resistance and, consequently, to type 2 diabetes, manifest diseases of the central nervous system (CNS) that may be influenced by disturbances of insulin signaling in the brain, such as memory impairment, Parkinson disease, and Alzheimer disease. We investigated the expression and response to insulin of elements involved in the insulin-signaling pathway in the forebrain cortex and cerebellum of rats ages 1 d to 60 wk. The protein content of insulin receptors and SRC homology adaptor protein (SHC) did not change significantly along the time frame analyzed. However, insulin-induced tyrosine phosphorylation of the insulin receptor and SHC, and the association of SHC/growth factor receptor binding protein-2 (GRB2) decreased significantly from d 1 to wk 60 of life in both types of tissues. Moreover, the expression of SH protein tyrosine phosphatase-2 (SHP2), a tyrosine phosphatase involved in insulin signal transduction and regulation of the insulin signal, decreased significantly with age progression, in both the forebrain cortex and the cerebellum of rats. Thus, elements involved in the insulin-signaling pathway are regulated at the expression and/or functional level in the CNS, and this regulation may play a role in insulin resistance in the brain.

Key Words: Insulin; SHC; GRB2; SHP2; central nervous system.

Introduction

Although a metabolic role for insulin in the central nervous system (CNS) has been contested on the basis of a lack of action on glucose uptake and metabolism in neuronal cells *(1)*, undisputed evidence demonstrates that insulin acts in the CNS, influencing feeding behavior by inducing satiety *(1)*. Receptors for insulin are present in several areas of the CNS with the greatest number being found in the olfactory bulb, paraventricular nucleus, arcuate nucleus, and ventromedial nucleus *(1–4)*. In these hypothalamic nuclei, insulin might act in concert with other regulators of feeding behavior, such as leptin, serotonin, corticotropic-releasing hormone and neuropeptide Y, in order to maintain the homeostasis of the organism, by regulating the ingestion of food, thermogenesis, and energy reserves *(5)*. Nevertheless, the presence of both insulin and elements of the insulin-signaling pathway in other areas of the CNS has evoked a series of studies attempting to characterize the action of the pancreatic hormone at such sites.

During rat embryogenesis, both insulin receptors (IRs) and insulin-like growth factor receptors (IGF-Rs) are highly expressed in several areas of the CNS and may play a role in growth and development *(6–8)*. Following birth, a progressive decrease in expression is observed for IRs in most regions of the brain; however, in the forebrain cortex, cerebellum, and several hypothalamic nuclei, IRs are still present at high concentrations during adulthood *(4,7–11)*. As for IGF-1R, both in the cerebellum and in several subareas of the forebrain, the binding of labeled IGF to brain slices increases slightly from birth to middle adulthood and then decreases progressively *(9,12,13)*. However, the levels of IGF-1R transcripts and protein are constant throughout life, with a subtle decrease in senile rats *(13)*.

Aging is a physiologic condition that leads to insulin resistance *(14–17)*. The mechanisms that link aging to progressive impairment of insulin action are not completely understood but involve a reduction in insulin-stimulated IR, insulin receptor substrate-1 (IRS-1), and insulin receptor substrate-2 (IRS-2) phosphorylation, followed by reduced IRS-1/-2-associated PI 3-kinase activity in the muscle and liver of rats *(18)*. One possible reason for the observed changes in the normal patterns of signal transduction through the IR-IRSs-PI 3-kinase pathway is a shift in tyrosine phosphatase activity, which may negatively modulate the timing of signaling through molecules activated by receptor tyrosine kinases *(19,20)*.

Although IRSs are present in the CNC, they are not phosphorylated in response to acute insulin treatment *(21,22)*. In fact, IRS-1 is not encountered in the same neurons expressing IR or IGF-1R *(22)*, suggesting that this docking protein may participate in the transmission of the signal generated by hormones or growth factors other than insulin or IGF-1.

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Table 1
Serum Insulin Levels (ng/mL) in Rats of All Groups

Age groups	D1	D7	D21	W6	W16	W60
Insulin (ng/mL) ±SEM	1.72 ±0.28[a]	1.21 ±0.33[b]	0.60 ±0.18[c]	0.62 ±0.11[c]	0.69 ±0.17[c]	1.19 ±0.30[b]

[a,b,c]Different letters represent statistical differences between groups, $p < 0.05$ ($n = 5$).

Nevertheless, acute insulin treatment stimulates IR and SRC homology adaptor protein (SHC) phosphorylation and SHC/growth factor receptor binding protein-2 (GRB2) association in the cerebellum but not in the forebrain cortex of adult rats *(23)*. Since levels of protein expression of elements involved in insulin signaling may suffer variations from embryonic life to senility, and supposing that this variation may play a role in the function of insulin in the CNS, the objective of the present study was to determine age-dependent variations in the expression of IR, SHC, GRB2, and SHP2 in the forebrain cortex and cerebellum of rats. IR, SHC, and SHC/GRB2 association were also evaluated for their responses to acute insulin treatment during different periods of life.

Results

Variation in Serum Insulin Level During Life

As depicted in Table 1, the level of serum insulin significantly falls from d 1 after birth to d 7 after birth, and from d 7 to d 21 after birth. Thereafter it is maintained constant from d 21 to wk 16 after birth and finally increases significantly from wk 16 to wk 60 after birth, reaching a level similar to that present in animals at d 7.

Expression of IR and SHC is Kept Constant Throughout Life

There is no significant variation in the protein amount of IR from birth to senility in the forebrain cortex and cerebellum of rats. As observed in Fig. 1A, the IR frequency is constant in the forebrain cortex while in the cerebellum there is a tendency to increase from d 1 to 7 and then it stabilizes up to wk 60 (Fig. 1B). The observed variation in the cerebellum is not significant. Similarly, the SHC protein concentration is unaffected by age, both in the forebrain cortex and in the cerebellum. As depicted in Figs. 1C,D, the constancy in SHC protein expression in forebrain cortex or in cerebellum is evident, and there is not even a tendency toward increased IR in the cerebellum during the first days of life.

Tyrosine Phosphorylation of IR Decreases During Life

When IR immunoprecipitates from saline or insulin-treated rats were analyzed for tyrosine phosphorylation, a significant and progressive decrease in the ratio of phosphorylation was detected in both the forebrain cortex (Fig. 2A) and the cerebellum (Fig. 2B). In both tissues, most variation occurred from d 1 to 7, and from d 7 to 21 and thereafter the rate of decrease was lower, and only in saline-treated IR precipitates from cerebellum was there again an accentuated decrease from wk 16 to 60 (Fig. 2A,B). Since no variation occurred in the protein amount of IR in the forebrain cortex or the cerebellum during the 60 wk of life evaluated, the observed reduction in phosphorylation was owing to reduced incorporation of phosphorus in tyrosine per IR molecule on a stoichiometric basis.

SHC Tyrosine Phosphorylation and SHC/GRB2 Association Decrease During Life

SHC immunoprecipitates blotted with anti-py antibodies revealed a progressive decrease in tyrosine phosphorylation in both saline and insulin-treated rats, in the forebrain cortex and in the cerebellum. As observed in Fig. 3A,B, a significant rate of decrease occurred from d 1 to 7, with a tendency of further reduction thereafter. Similar to what was observed for IR, the decrease in phosphorylation of SHC was owing to reduced incorporation of phosphorus per SHC molecule and not to a reduction in the SHC concentration.

Insulin-induced association of SHC and GRB2 decreased significantly with age, both in the forebrain cortex and in the cerebellum (Fig. 4A,B). The rate of decrease was similar to that observed for SHC tyrosine phosphorylation. Once again, as SHC and GRB2 protein levels (data not shown) remained stable during the life span analyzed, the observed reduction in SHC/GRB2 association induced by insulin was a specific phenomenon linked to aging and not dependent on substrate availability.

SHP2 Levels Decrease During Life

The tyrosine phosphatase SHP2 may play an important role as a modulator of hormone and growth factor signaling through receptor tyrosine kinases. Because levels of phosphorylation of IR and SHC decreased progressively during life in both the forebrain cortex and cerebellum of rats in spite of the constancy of the protein amounts of both signalers, we decided to study the protein levels of SHP2 at various ages in the CNS of rats. In both the forebrain cortex and the cerebellum, the levels of SHP2 fell significantly between d 1 and wk 60. From d 1 to 7 a subtle increase in the amount of protein was observed, and from d 7 to wk 6 (wk 6 after birth) there was a significant fall, which was maintained until wk 60 (Fig. 5A,B).

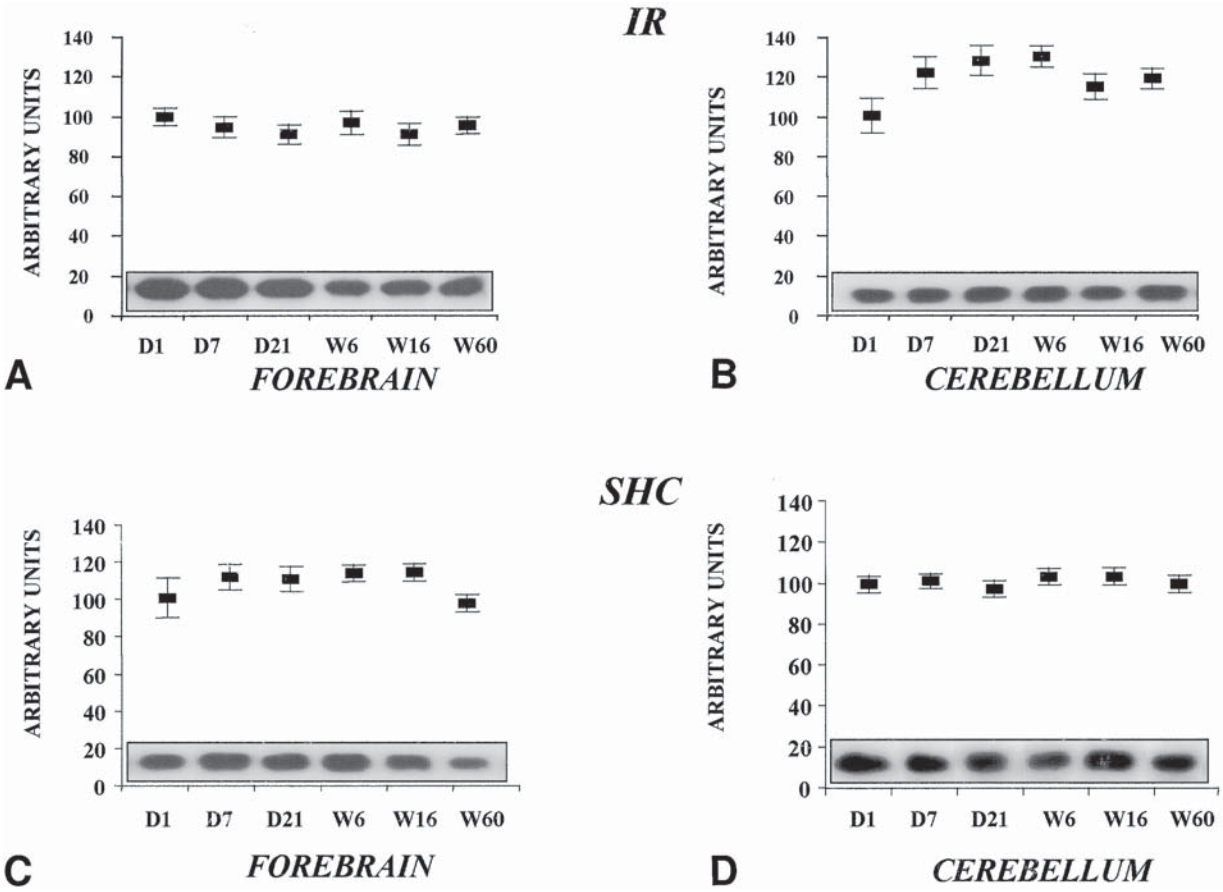

Fig. 1. IR and SHC expression are kept constant during life. Proteins from the forebrain cortex and cerebellum were analyzed as described in Materials and Methods. Briefly, rats were anesthetized and fragments of each tissue were excised and homogenized in extraction buffer A at +4ºC. After centrifugation, aliquots of the homogenate supernatants containing equal amounts of protein were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were subsequently transferred to nitrocellulose membranes and detected by immunoblotting (IB) with anti-IR (**A,B**) or anti-SHC (**C,D**) antibodies and ^{125}I-protein A, after which the membranes were autoradiographed. No statistically significant difference was detected between ages (n = 8). The scans of the blots are representative images obtained from a single experiment.

Discussion

Insulin action in target cells begins with the binding of this hormone to the α-subunit of the IR, which leads to the activation of tyrosine kinase activity in the transmembrane β-subunit *(24–26)*. Once activated, the IR undergoes autophosphorylation and subsequently recruits a series of intracellular substrates that will act as docking proteins, directing the insulin message toward specific subcellular compartments and thus modulating cell behavior *(27)*. The main docking proteins of the insulin signaling pathway are the insulin receptor substrates 1 and 2 (IRS 1 and IRS 2), and SHC *(27,28)*. The IRS proteins are apparently specialized in transmitting the insulin and the IGF-1 signal, and only a few other hormones have been shown to activate this pathway *(27,29,30)*. The same is not true for SHC, which is an ubiquitous protein expressed as three different isoforms (46, 52, and 66 kDa), and enrolled as an intracellular signal transducer in several signaling pathways *(31–33)*. SHC is phosphorylated by the IR through a phosphotyrosine-binding domain located at its amino-terminus *(34,35)*. Once phosphorylated, SHC binds to the GRB2-son-of-sevenless (SOS) complex inducing the relocation of SOS to a juxtamembrane intracellular compartment. At this site, SOS promotes the exchange of guanosine 5'-diphosphate to guanosine 5'-triphosphate, associated with Ras, and through this mechanism activates Ras, which will lead to mitogen-activated protein kinase (MAPK) activation and stimulation of cellular growth and mitogenesis *(34)*.

Insulin action and signal transduction has been mostly evaluated in liver, muscle, and adipose tissue, sites of prevailing metabolic importance for the organism *(18,36,37)*. Until recently, only a few studies have focused on the roles

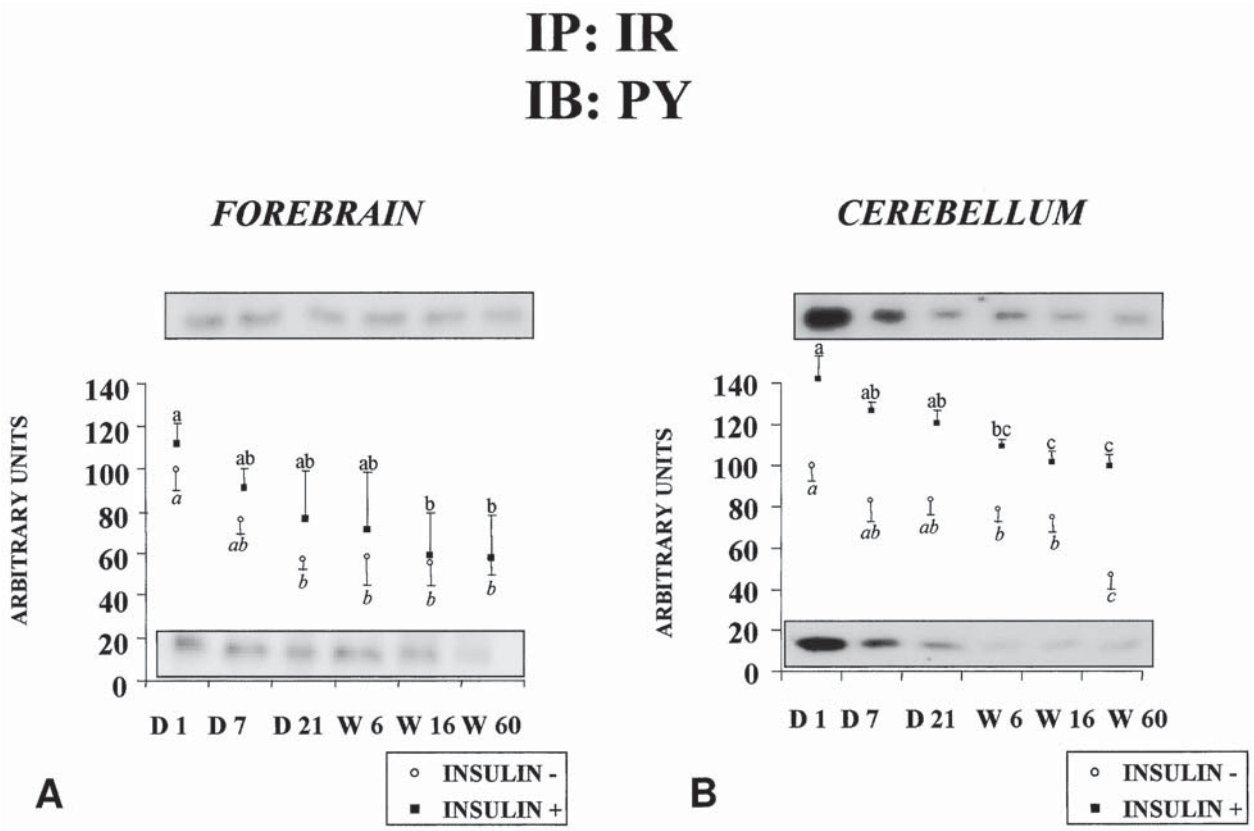

Fig. 2. Tyrosine phosphorylation of IR decreases during life. Forebrain cortex and cerebellum were extracted and homogenized as described in Materials and Methods, after iv injection of saline (–) or insulin (+). Aliquots of the homogenate supernatants containing equal amounts of protein were immunoprecipitated (IP) with anti-IR, and the immunocomplexes were collected with protein A-Sepharose 6MB and then resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and detected with antiphosphotyrosine antibody (py) (**A,B**) and ^{125}I-protein A followed by autoradiography. Different letters represent statistically significant differences between groups ($p < 0.05$; $n = 10$). The scans of the blots are representative images obtained from a single experiment.

of insulin in the CNS. Because neuronal cells do not depend on insulin to internalize glucose, diabetologists and endocrine physiologists emphasized their studies on the traditional targets of insulin action. However, since 1978, with the description of insulin *(38)* and insulin receptors *(39)* in the brain, a rapidly increasing number of publications have been dealing with the characterization of insulin activity in the CNS. We already have learned that IR is widely distributed in the brain, with the highest concentration in the hypothalamus; that IRS-1 is present in the CNS but does not coincide with the distribution of IR or IGF-1R *(7)*; and that SHC, GRB2, SHP2, and elements of the MAPK signaling pathway are also expressed in various brain regions *(23,40)*.

In the present study, age-dependent variations in the expression of various elements of the insulin-signaling pathway were analyzed in the forebrain cortex and cerebellum. A few previous studies have evaluated the variations of IR in the brain; most of these analyzed binding sites *(9,11,41)* and not protein content *(7)*. When analyzing binding sites for insulin, most researchers have detected a progressive decrease from birth to senility *(13)*; however, Kappy and Raizada *(10)* found that working with purified rat brain membranes, there were no differences in insulin-binding sites between term fetus and adults. Investigation of IGF-1-binding sites in the brain follows the same pattern, with most researchers finding a progressive decrease from birth to adulthood *(13)*. In the forebrain cortex and cerebellum extracts evaluated, we found no significant changes in the protein amounts of IR and IGF-1R (data not shown) from d 1 to wk 60 in male Wistar rats. In an extensive review of the literature, we found only one study in which IR and IGF-1R expression were evaluated using immunoreactive methods *(7)*; Garofalo and Rosen *(7)*, using a panel of antibodies crossreactive with the β-subunit of IR and IGF-1R, describe a progressive fall in immunoreactive forms of both receptors, from embryonic life to adulthood. Differently from the methodology employed in our study, they used nonspecific antibodies, performed blots in total brain extracts, and based their conclusions on the densitometric quantitations of only two experiments. As for a reduction in insu-

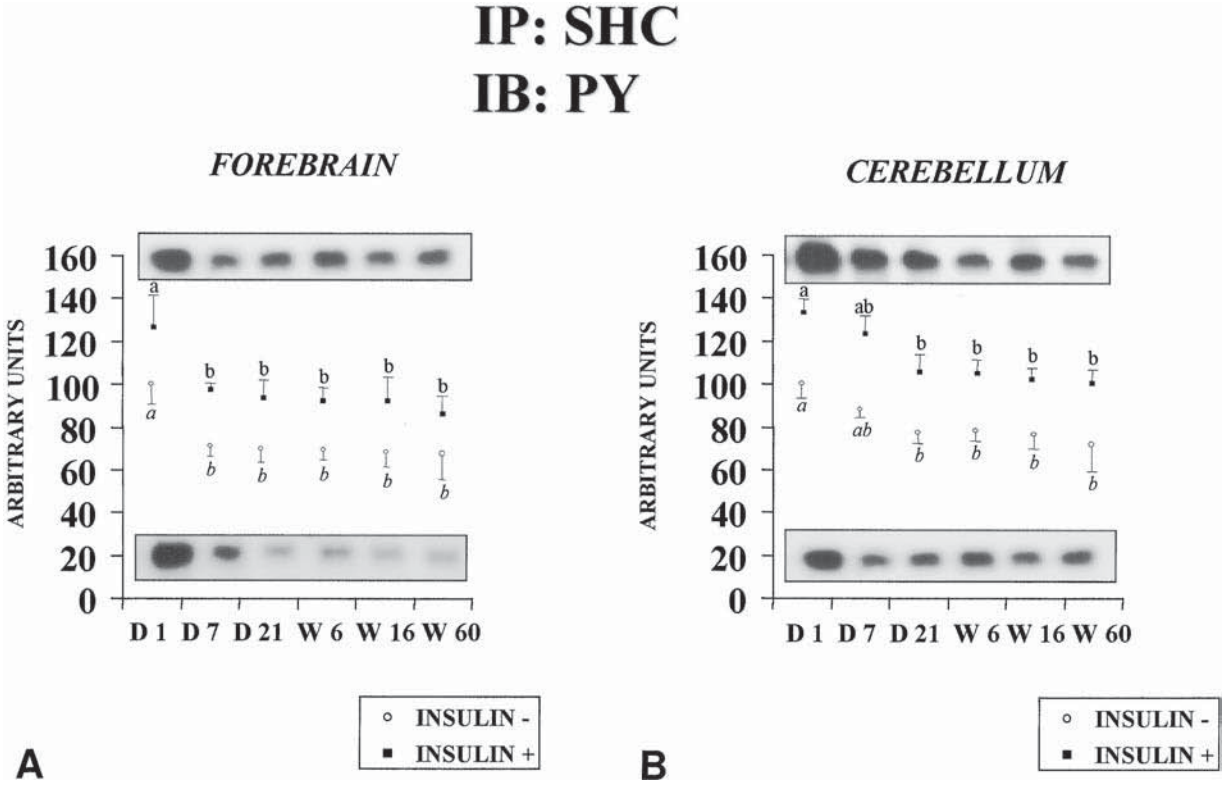

Fig. 3. SHC tyrosine phosphorylation decreases during life. Forebrain cortex and cerebellum samples were extracted and homogenized as described in Materials and Methods. Saline (–) or insulin (+) was administered intravenously as a bolus injection. Aliquots of homogenate supernatants containing equal amounts of protein were immunoprecipitated (IP) with anti-SHC. Immunoprecipitates were resolved on SDS-PAGE. The proteins were transferred to nitrocellulose membranes and detected with py (**A,B**) and ^{125}I-protein A, followed by autoradiography. Different letters represent statistically significant differences between groups ($p < 0.05$; $n = 10$). The scans of the blots are representative images obtained from a single experiment.

lin and IGF-1-binding sites described in most studies, we believe that it serves as an evaluation of available binding sites at the cell surface and may be affected by the dynamics of receptor cycling from internal pools to the membrane. With the quantitative methods employed in the present study, we found that from d 1 after birth to wk 60 of life (incipient senility), there were no significant changes in the expression of IR in the forebrain cortex and cerebellum of rats.

Similar to IR, no significant variations occurred in the expression of the protein SHC in either of the tissues we analyzed. In recent studies, Cattaneo and Pellicci *(34)* and Conti et al. *(42)* describe a much higher expression of SHC during embryonic life than in adulthood. However, no evaluation of variability during postnatal lifetime was made.

When analyzing the tyrosine-phosphorylated forms of IR and SHC, and the magnitude of SHC/GRB2 association in the forebrain cortex and cerebellum of saline or insulin-treated rats, a progressive fall from d 1 to wk 60 was encountered in every situation studied. When, instead of comparing absolute levels of insulin-induced protein tyrosine phosphorylation or protein-protein binding, we compared fold stimulation between basal and insulin-treated conditions, no significant difference was detected between age groups (data not shown). However, in tissues that act as traditional targets for insulin action, it appears that total tyrosine phosphorylation of IR and SHC, and SHC-GRB2 association (and not fold stimulation), are the molecular events most closely related to the final physiologic effect of insulin. In the anatomic sites evaluated in our study, no known physiologic events controlled by insulin are measurable, but we suspect that the progressive fall in insulin-stimulated molecular response may be linked to a loss of function of insulin during aging in the forebrain cortex and cerebellum. Demonstration of a rise in serum insulin level from wk 16 to 60 would further support this hypothesis. Thus, since activity of the SHC-GRB2 branch of the insulin-signaling pathway depends on SHC tyrosine phosphorylation and SHC/GRB2 association, we believe that aging may induce impairment of signal transduction through this channel. The signal driven

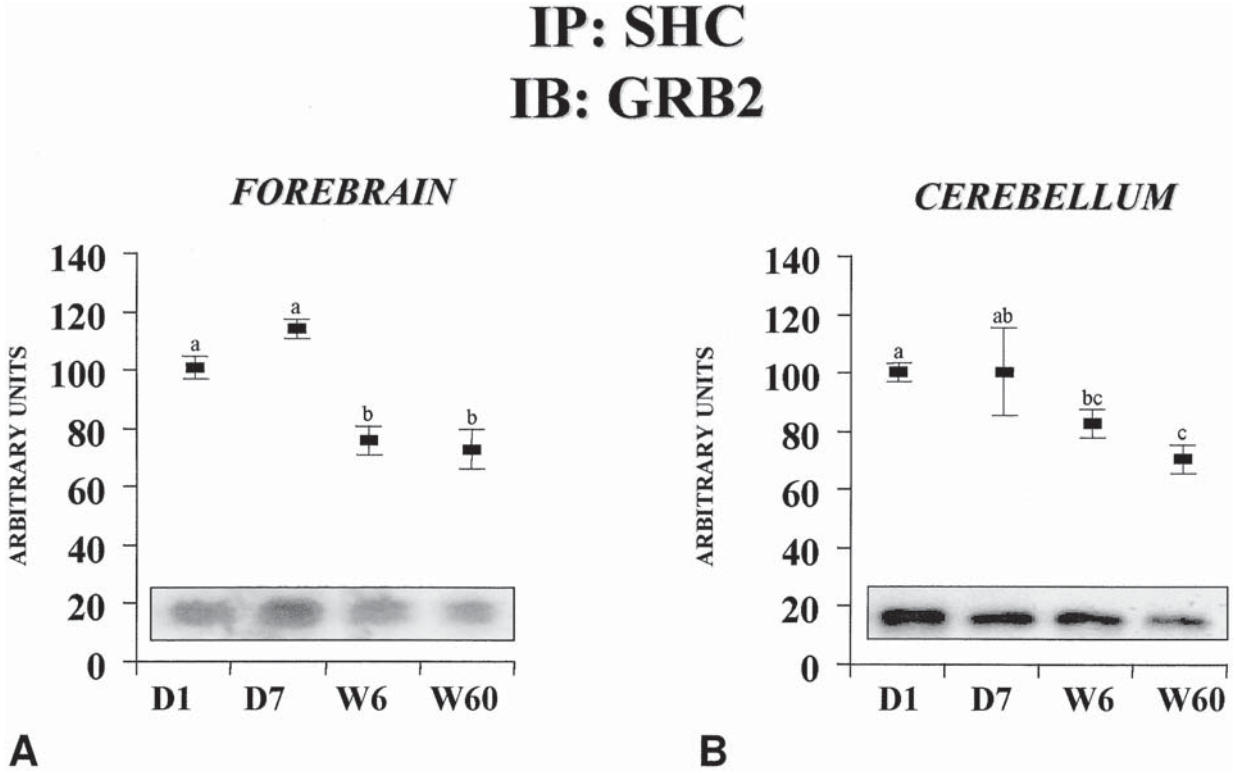

Fig. 4. SHC/GRB2 association decreases during life. Forebrain cortex and cerebellum samples were extracted and homogenized as described in Materials and Methods. Saline (–) or insulin (+) were administered intravenously as a bolus injection. Aliquots of homogenate supernatants containing equal amounts of protein were immunoprecipitated (IP) with anti-SHC and resolved on SDS-PAGE. The proteins were transferred to nitrocellulose membranes and detected with anti-GRB2 antibodies and ^{125}I-protein A, followed by autoradiography (**A,B**). Different letters represent statistically significant differences between groups ($p < 0.05$; $n = 10$). The scans of the blots are representative images obtained from a single experiment.

by SHC/GRB2 and SOS leads to Ras and MAPK activation and positively modulates multiple cellular events linked to development, growth, and mitogenesis.

Impairment of insulin-stimulated phosphorylation of IR, IRS-1/-2, and IRSs/PI 3-kinase association/activation has been described in muscle and liver of aging rats *(18)*. The results of those experiments and other reports *(15,18,19)* suggest that increased tyrosine phosphatase activity in older rats could be one of the mediators of such observations, and that these phenomena could be implicated in the well-known insulin resistance observed in aging animals or humans *(14–16)*. Because the tyrosine phosphatase SHP2 is widely expressed in CNS, and is a potential regulator of insulin signaling in brain, we measured SHP2 protein expression during life in the forebrain cortex and cerebellum of rats and found that after a subtle increase from d 1 to 7 the level of protein decreased progressively until wk 60. Although no study of direct interaction between SHP2 and the insulin-stimulated SHC/GRB2 pathway was made, the progressive fall of SHP2 expression in both tissues, occurring in parallel with the decrease in IR and SHC phosphorylation, and SHC/GRB2 association is suggestive of a mechanistic involvement.

In conclusion, during the animals' lifetime, the amounts of IR and SHC remained constant in rat forebrain cortex and cerebellum, while basal and insulin-stimulated IR and SHC phosphorylation, and SHC/GRB2 association underwent a progressive, age-dependent fall. During the same period of evaluation, the levels of SHP2 protein fell progressively.

Materials and Methods

Antibodies, Chemicals, and Buffers

Antibodies against IR, SHC, GRB2, and SHP2 were from Santa Cruz Biotechnology (Santa Cruz, CA). The SHC antibodies utilized recognize both the 46- and 52-kDa isoforms, and by molecular mass analyses it was determined that the major isoform expressed in postnatal CNS is SHC-A 52. Thus, when referring to SHC we mean SHC-A 52 kDa. Antibodies against phosphotyrosine (py) were from UBI (Lake Placid, NY). A rat insulin radioimmunoassay (RIA) kit was purchased from Linco (St. Charles, MO). ^{125}I-Protein A Sepharose was from Amersham (Buckinghamshire, UK). Protein A Sepharose 6MB was from Pharmacia (Uppsala, Sweden). All the remaining chemicals used in the experiments were from Sigma (St. Louis, MO). Buffer A consisted

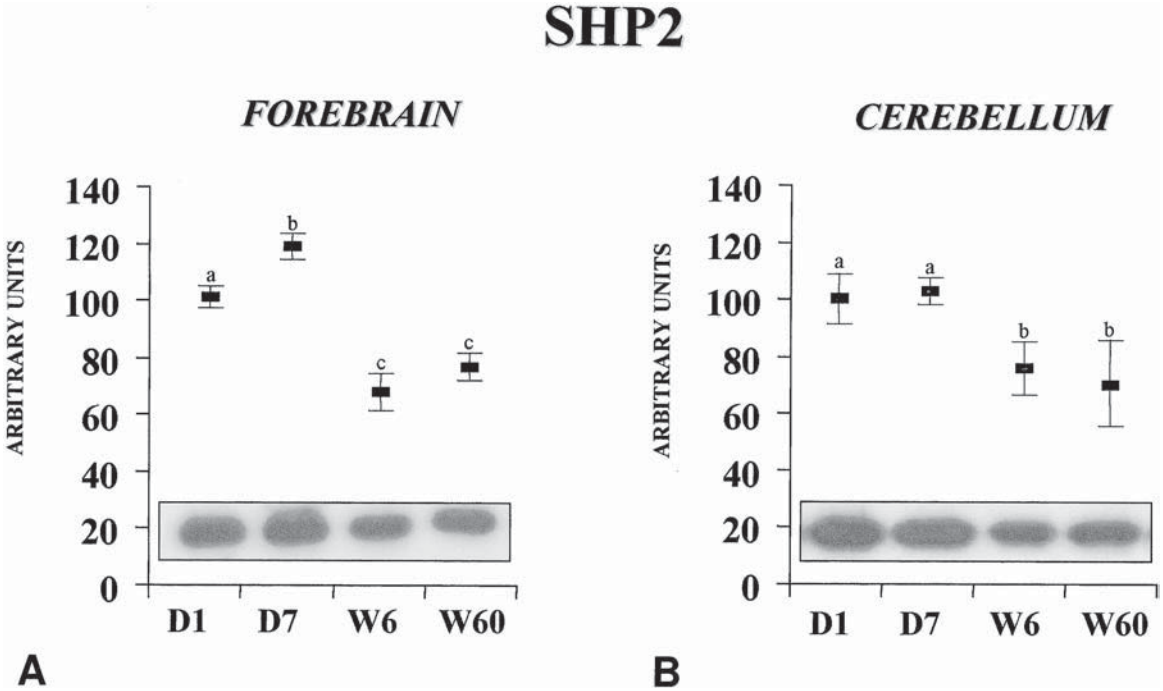

Fig. 5. SHP2 levels decrease during life. Proteins from the forebrain cortex (**A**) and cerebellum (**B**) were analyzed as described in Materials and Methods. Briefly, rats were anesthetized and fragments of each tissue were excised and homogenized in extraction buffer A at +4ºC. After centrifugation, aliquots of the homogenate supernatants containing equal amounts of protein were resolved on SDS-PAGE. The protein bands were subsequently transferred to nitrocellulose membranes and detected by immunoblotting (IB) with anti-SHP2 antibodies and ^{125}I-protein A, followed by autoradiography. Different letters represent statistically significant differences between groups ($p < 0.05$; $n = 7$). The scans of the blots are representative images obtained from a single experiment.

of 100 m*M* Tris, 1% (v/v) Triton X-100, 50 m*M* HEPES (pH 7.4), 100 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride, 10 m*M* EDTA, 10 m*M* sodium vanadate, 2 m*M* phenylmethylsulfonyl fluoride, and 0.1 mg/mL of aprotinin. Buffer B consisted of 100 m*M* Tris, 10 m*M* sodium vanadate, 10 m*M* EDTA, and 1% Triton X-100.

Animals

All experiments were performed with male Wistar rats from the University of Campinas Breeding Center. Animals were studied at six different ages; d 1, d 7, d 21, wk 6, wk 16, and wk 60 after birth. Day one and d 7 rats were nursed by their progenitors, while the remaining rats were fed standard rodent chow and water ad libitum. The experimental animals were housed under a 12-h light and dark cycle at 23ºC. Day 1 and 7 rats were separated from their progenitors 2 h before the experiments; the remaining rats were food deprived 6–8 h before the experimental procedure. All experiments were approved by the University of Campinas Ethical Committee.

Determination of Serum Insulin

Five rats from each group were selected for venous blood collection for insulin measurement. Samples were taken after a 6-h fast. Serum insulin was measured utilizing a commercial RIA kit from Linco, following the manufacturer's recommendations.

Experimental Procedures

Rats were anesthetized by ip injection of sodium amobarbital (15 mg/kg of body wt) and experiments started after the loss of corneal and pedal reflexes. For specific protein determination experiments, 3 × 3 mm fragments of forebrain cortex or cerebellum cortex were excised, homogenized in ice-cold buffer A, and the insoluble material was removed by centrifugation for 45 min (50,000*g*) at +4ºC. Protein quantification in the supernatants was determined by the Bradford method *(43)*. Samples containing 0.2 mg of total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific antibodies. For evaluation of insulin-induced protein phosphorylation or association, experimental animals had their abdominal cavity opened and insulin (500 µg/kg of body wt, diluted 120 µg/mL in saline) or saline alone (same volume/kg of body wt as the insulin solution) was injected. Three minutes after injection (optimal time as previously determined) *(23)*, an approx 3 × 3 mm fragment was excised

from the forebrain cortex or from the cerebellum cortex and immediately homogenized in buffer A at +4ºC. Insoluble material was removed by centrifugation for 45 min (50,000*g*) at +4ºC. Protein quantification in the supernatants was determined by the Bradford method *(43)*. Samples containing 3 mg of total protein were used in immunoprecipitation experiments with anti-IR or anti-SHC antibodies. Immunoprecipitates were collected with protein A Sepharose 6MB, repeatedly washed with ice-cold buffer B, and resolved by SDS-PAGE, followed by transfer and blotting with specific antibodies. IR immunoprecipitates were blotted with anti-IR antibodies and with anti-py antibodies, while SHC immunoprecipitates were blotted with anti-SHC antibodies, anti-py antibodies, and anti-GRB2. Blots were visualized by incubating membranes with ^{125}I-protein A and exposing to RX-films. For each set of experiments, samples collected from rats of each of the age groups were run side by side in the same gel.

Statistical Analyses

Specific protein bands present in the blots were quantified by densitometry. Mean values ± SEM obtained from densitometric scans and values for serum insulin were compared utilizing students's *t*-test for unpaired data. A value of $p < 0.05$ was considered as statistically significant.

Acknowledgments

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