Neurochemical Research, Vol. 6, No. 5, 1981

EFFECT OF HYPOXIA ON NUCLEIC ACID AND PROTEIN SYNTHESIS IN DIFFERENT BRAIN REGIONS

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Accepted January 20, 1981

The incorporation of [*methyl*-^{3}H]thymidine into DNA, of [5-^{3}H]uridine into RNA, and of [1-^{14}C]leucine into proteins of cerebral hemispheres, cerebellum, and brainstem of guinea pigs after 80 hr of hypoxic treatment was measured. Both in vivo (intraventricular administration of labeled precursors) and in vitro (tissue slices incubation) experiments were performed. The labeling of macromolecules extracted from the various subcellular fractions of the above-mentioned brain regions was also determined. After hypoxic treatment the incorporation of the labeled precursors into DNA, RNA, and proteins was impaired to a different extent in the three brain regions and in the various subcellular fractions examined; DNA and RNA labeling in cerebellar mitochondria and protein labeling in microsomes of the three brain regions examined were particularly affected.

INTRODUCTION

It is well known that the central nervous system is very sensitive to oxygen deprivation due to its great energy requirement. Cerebral hypoxia results in a series of biochemical and physiological events which cause a rapid and irreversible loss of neuronal function (9, 11, 21, 23). Electron microscopic investigations (24, 28, 36) showed early cellular alterations soon after a lack of oxygen.

Morphological and biochemical studies (6, 17, 41) indicated that neuronal and glial cells are affected to a different extent by cerebral hypoxia, anoxia, or ischemia.

Up to now, the metabolic changes reported taking place in nervous

0364-3190/81/0500-0595$03.00/0

tissue following O_2 deprivation are mainly concerned with the oxidative metabolism of carbohydrates (7, 26, 27). A decrease of the oxidative phosphorylation rate and a low level of high-energy compounds (5, 9, 27, 29) have also been reported in hypoxic conditions. However, to our knowledge, very few studies have been performed on nucleic acid and protein metabolism in brain tissue during hypoxia (6, 8, 34, 39).

In the present study, we measured the incorporation of labeled precursors into DNA, RNA, and proteins in different regions and in various subcellular fractions of guinea pig brain during hypoxia. Both in vivo (intraventricular administration of labeled precursors) and in vitro (tissue slices) experiments were carried out.

EXPERIMENTAL PROCEDURE

Materials. [*methyl*-^{3}H]Thymidine (specific activity 2 Ci/mmol), [5-^{3}H]uridine (specific activity 5 Ci/mmol); [1-^{14}C]leucine (specific activity 59 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England). Ficoll was purchased from Pharmacia Fine Chemicals, Inc. (Sweden) and used without any treatment. Other solvents and reagents were of analytical grade.

Animals and Hypoxic Treatment. For the experiments, adult female guinea pigs (400 g body weight) were fasted for 12 hr. Hypoxic treatment consisted of keeping the animals intermittently (17 hr daily), for a total period of 80 hr, in a chamber flushed with a mixture of oxygen and nitrogen (9:91), as previously described (1).

Surgical Operation. A local anaesthesia was produced by means of a subcutaneous injection of carbocaine solution under the scalp; a midline incision was then made on the animal's scalp. A small hole was made in the skull over the lateral ventricle using a dental drill filled with polyethylene sleeve. A plastic cannula, filled with saline was tightly inserted into the hole, sealed by a flame, and fixed in the skull using dental cement. The scalp incision was closed with surgical clips and the animals were transferred to cages or to hypoxic chambers. Each animal received, through the reopened cannula (using a 50-μl Hamilton syringe), an injection of either single or combined precursors. All injections were given slowly, to avoid leakage of the isotope solution. The cannula was washed with 10 μl of saline and sealed again with a flame. No antibiotics were given to the animals.

In Vivo Assays. The animals which received an intraventricular administration of the labeled precursors, had a surgical operation as described above. Twelve hr later the animals received an injection of [*methyl*-^{3}H]thymidine (5 μCi/g brain weight), [5-^{3}H]uridine (5 μCi/g brain weight), and [1-^{14}C]leucine (1 μCi/g brain weight), either combined or separately, into the lateral ventricle. Ninety min after the injection, the animals were killed by decapitation.

The time of sacrifice was chosen on the basis of preliminary experiments on time course in the different brain regions and subcellular fractions indicating that the incorporation of the three labeled precursors was linear up to 90 min.

The brains were rapidly removed and dissected in the cold room into three regions: cerebral hemispheres, cerebellum, and brainstem. Homogenates were prepared as described below.

In Vitro Assays. Tissue slices (0.3 mm thick), obtained with a McIlwain and Buddle tissue chopper (42), were incubated at 37°C in a shaking water bath in Krebs-Ringer phosphate buffer, pH 7.4, gased with O_2–CO_2 (95:5), containing glucose (10 mM) and the following labeled precursors (either combined or separately): [5-*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, and [1-^{14}C]leucine (1 μCi of each precursor/g wet weight in 3 ml of incubation medium). All assays were run in duplicate or triplicate. The incubation time was 1 hr, according to the kinetics of the labeling previously established (13). At the end of the incubation period, homogenates were prepared and subcellular fractions were separated as described below.

Preparation of Homogenates and Separation of Subcellular Fractions. Homogenates were prepared with 4 vol of 0.32 M sucrose containing 3 mM $MgCl_2$ and 0.1 mM potassium phosphate buffer, pH 6.4 (medium A). Homogenates were diluted 1:20 with medium A and centrifuged at 1000 *g* for 10 min. The supernatants were saved for separation of the mitochondria. The pellets were washed with medium A and centrifuged again as above. The nuclear pellet was purified according to the technique used in previous studies (14). This mainly consists in the resuspension of nuclei in 2.4 M sucrose solution, containing 1 mM $MgCl_2$ (2 mM for cerebellum) and 1 mM potassium phosphate buffer, pH 6.4 (the final concentration of sucrose was adjusted to 2 M by refractometry); the suspension was centrifuged for 1 hr at 75,000 *g* in the SW 27 rotor of a Spinco ultracentrifuge model L2-65.

Mitochondria were isolated following Goldberg's technique (15), with slight modifications (12, 13). The postnuclear supernatant was centrifuged at 12,000 *g* for 30 min. The pellet was resuspended in 4 ml of 0.32 M sucrose, containing 1 mM EDTA and 10 mM Tris HCl buffer, pH 7.4, and centrifuged for 1 hr at 65,000 *g* in a Spinco SW 27 rotor, through the following layers of a Ficoll gradient in medium A: 12%, 10%, 8%, 5% (7 ml of each) from the bottom to the top.

The supernatants, obtained after mitochondrial separation, were centrifuged at 105,000 *g* for 2 hr to obtain the microsomal fraction; the remaining supernatants were taken as the cytoplasmic-soluble fraction.

Purity of Subcellular Fractions. The purity of mitochondrial preparations was checked by electron microscopy and by using specific inhibitors of mitochondrial DNA, RNA, and protein synthesis, as in previous studies (12). In order to control the degree of purity in the mitochondrial preparation, the activities of succinate dehydrogenase (EC 1.3.99.1) as mitochondrial marker and of 5′-nucleotidase (EC 3.1.3.5) as plasma membrane marker were always determined. Contamination of mitochondrial preparation was never more than 5–10% and was generally represented by a few nerve endings.

The purification of nuclei has been performed as in previous studies (14), according to the technique of Austoker et al. (3) by which nuclei presenting very little visible cytoplasmic contamination and usually preserving their morphological characteristics can be obtained (as demonstrated by phase-microscopic examination of the nuclear preparation).

Extraction of DNA, RNA, and Proteins. DNA, RNA, and proteins from nuclei and mitochondria, RNA and proteins from microsomes, and cytoplasmic-soluble fractions were extracted according to the method of Schmidt and Tannhauser (33) with some modifications (13). The acid-soluble compounds were removed from each subcellular fraction by several washings with cold 0.5 N $HClO_4$, until no further radioactivity could be detected in the washing solution. The precipitate was washed twice with each of the following solvents: acetone, ethanol, ether, and successively dried at room temperature in air. The resulting powders were resuspended in a small volume (0.1–0.5 ml, depending on the amount of the precipitate) of 0.3 N KOH and incubated at 37°C for 18 hr in order to hydrolyze RNA to mononucleotides. After that, DNA and proteins were precipitated by addition, at 4°C, of

60% (w/v) $HClO_4$, drop by drop, to pH 1. The sediment was washed twice with a small volume of cold 0.2 N $HClO_4$, and the combined supernatants were employed for RNA estimation and for radioactivity measurements. After several washings of the pellet with cold 0.2 $HClO_4$, DNA was extracted twice with 0.5 N $HClO_4$, at 70°C. Aliquots of this solution were employed for DNA estimation and for the measurement of radioactivity. DNA and RNA were determined spectrophotometrically according to Wannemacher et al. (41). Proteins were extracted and the specific radioactivity was determined according to the technique employed in previous studies (13). The results are expressed as dpm/mg DNA, RNA, or protein (mean ± SEM).

Determination of Radioactivity. All the samples were counted using Instagel (Packard) liquid scintillator. The radioactivity was measured by a Nuclear Chicago scintillation counter, model Isocap 300; the counts were corrected for 100% efficiency.

RESULTS

Effect of Hypoxia on DNA Labeling. The results of in vivo and in vitro incorporation of [5-*methyl*-^{3}H]thymidine into DNA extracted from total tissue (TT), nuclei (N), and mitochondria (Mt) of cerebral hemispheres, cerebellum, and brainstem of control and hypoxic animals are shown in Figure 1.

The specific radioactivity of DNA significantly decreased in all brain regions of hypoxic animals compared to the controls. Both nuclear and mitochondrial DNA labeling decreased after the hypoxic treatment; however, mitochondrial DNA was more affected by hypoxia, especially in the in vivo experiments.

The incorporation of [^{3}H]thymidine into mitochondrial DNA was significantly higher than that into nuclear DNA in both hypoxic and control animals, in agreement with our previous findings, showing a high biosynthesis of mitochondrial DNA in adult brain (12, 13).

Effect of Hypoxia on RNA Labeling. The results of the in vivo and in vitro incorporation of [^{3}H]uridine into RNA extracted from total tissue, as well as from subcellular fractions, namely nuclei (N), mitochondria (Mt), microsomes (Mc), and soluble fraction (SF) from the three above-mentioned brain regions of control and hypoxic animals are reported in Figure 2. Quite similar results were obtained in both the in vivo and in vitro experiments.

The specific radioactivity of RNA significantly decreased in all brain regions of hypoxic animals compared to the controls. RNA labeling in the various subcellular fractions was affected to a different extent by the hypoxic treatment. The decrease of [^{3}H]uridine incorporation into RNA was significant in some fractions, as indicated by the statistical analysis reported in Figure 2. Mitochondrial RNA labeling in the cerebellum was particularly impaired by the hypoxic treatment.

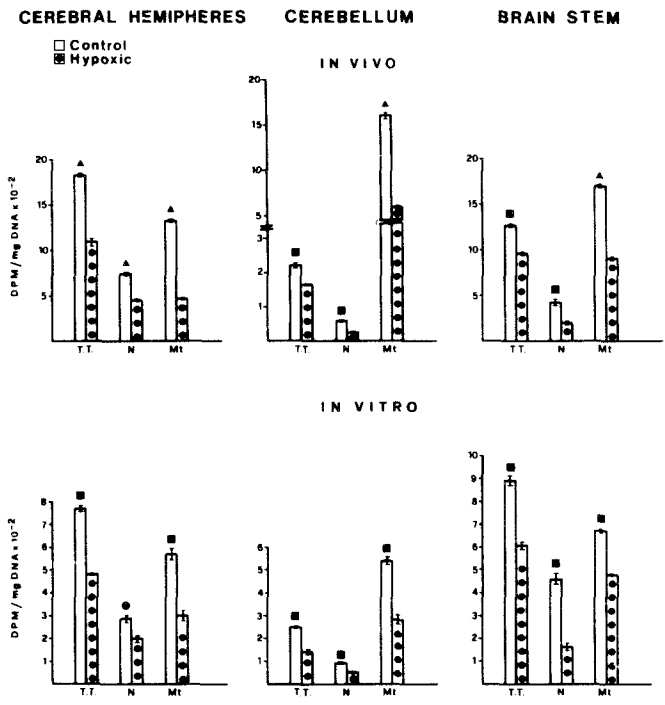

FIG. 1. Effect of hypoxia on the in vivo and in vitro incorporation of [*methyl*-^{3}H]thymidine into DNA extracted from total tissue (TT), nuclei (N), and mitochondria (Mt) of cerebral hemispheres, cerebellum, and brainstem of guinea pigs. The results are expressed as specific radioactivity (dpm/mg DNA) and represent the average of three independent experiments with four animals in each group. Vertical bars represent the SEM. The degree of significance of the differences between the mean values obtained for control and hypoxic animals was analyzed by Student's *t* test. The *P* is indicated as follows: ◣ = not significant; ● = $P < 0.05$; ■ = $P < 0.01$; ▲ = $P < 0.001$.

Effect of Hypoxia on Protein Labeling. The results of [1-^{14}C]leucine incorporation into proteins extracted from total tissue and from the different subcellular fractions of cerebral hemispheres, cerebellum, and brainstem of control and hypoxic animals are shown in Figure 3.

The specific radioactivity of proteins significantly decreased in the brain regions of hypoxic animals compared to the controls, with the exception of that in the brainstem in the in vivo experiments. The decrease of [1-^{14}C]leucine incorporation into proteins of subcellular fractions examined was significant in some of them, as indicated by the statistical analysis reported. The most marked effect of hypoxic treatment on protein labeling

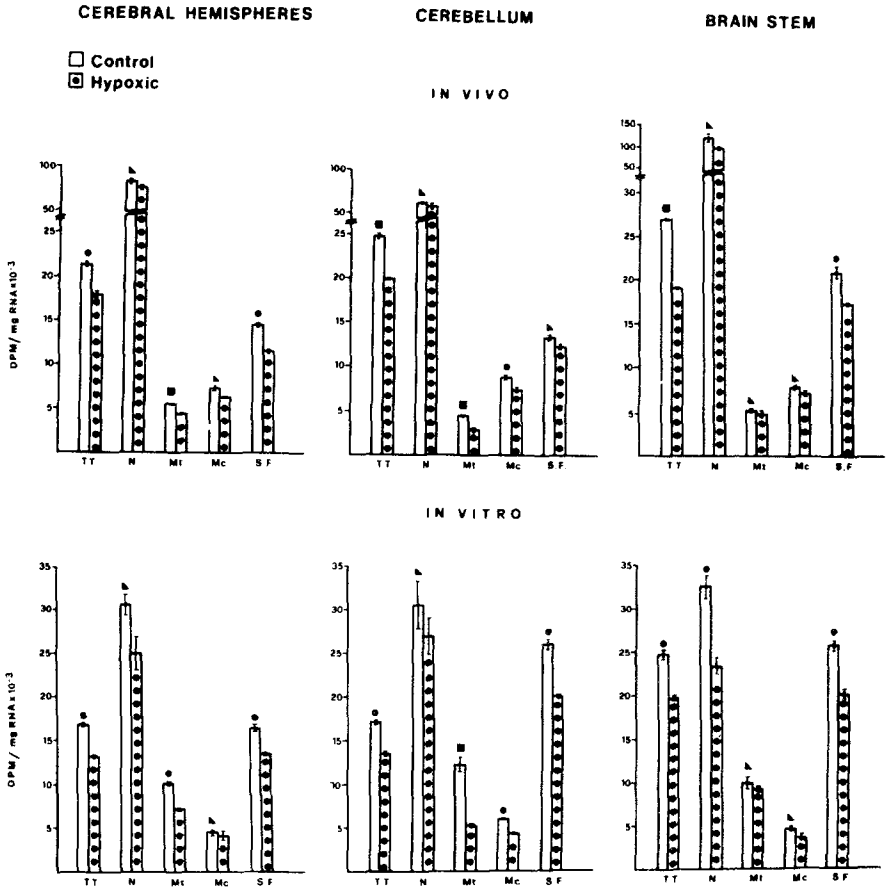

FIG. 2. Effect of hypoxia on the in vivo and in vitro incorporation of [5-^{3}H]uridine into RNA extracted from total tissue (TT) and from the various subcellular fractions (nuclei, N; mitochondria, Mt; microsomes, Mc; soluble fraction, SF) of cerebral hemispheres, cerebellum and brainstem of guinea pigs. The results are expressed as specific radioactivity (dpm/mg RNA) and represent the average of three independent experiments with four animals in each group. Vertical bars represent the SEM. The degree of significance of the differences between the mean values obtained for control and hypoxic animals was analyzed by the Student's t test. The P value is indicated as follows: ◣ = not significant; ● = $P < 0.05$; ■ = $P < 0.01$; ▲ = $P < 0.001$.

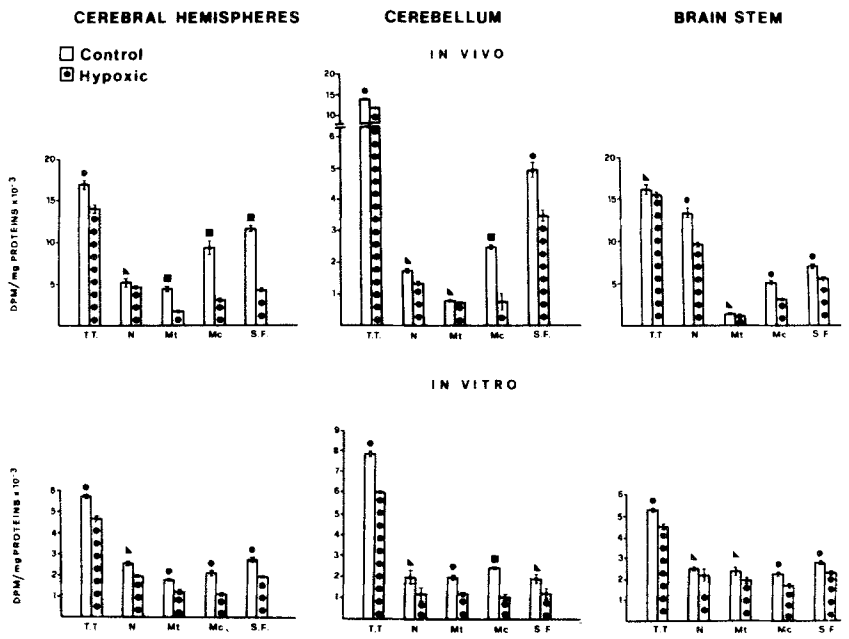

FIG. 3. Effect of hypoxia on the in vivo and in vitro incorporation of [1-^{14}C]leucine into proteins extracted from total tissue (TT) and from the various subcellular fractions (nuclei, N; mitochondria, Mt; microsomes, Mc; soluble fraction, SF) of cerebral hemispheres, cerebellum, and brainstem of guinea pigs. The results are expressed as specific radioactivity (dpm/mg protein) and represent the average of three independent experiments with four animals in each group. Vertical bars represent the SEM. The degree of significance of the differences between the mean values obtained for control and hypoxic animals was analyzed by the Student's t test. The P value is indicated as follows: ◣ = not significant; ● = $P < 0.05$; ■ = $P < 0.01$; ▲ = $P < 0.001$.

was observed in the microsomal fraction of the three brain regions examined in both in vivo and in vitro experiments.

DISCUSSION

One of the main difficulties in studying the effect of hypoxia in the various brain regions is due to the difference in the rate of regional blood flow (21, 31). This might diversely affect the in vivo uptake of a labeled precursor from the blood. In an attempt to overcome this problem, we performed experiments both in vivo, by injecting the labeled precursors directly into the lateral ventricle, and in vitro with tissue slices. These

last offer the advantage of keeping the tissue under stable conditions during the incubation time.

The results obtained in the present study indicated that hypoxic treatment markedly impairs DNA, RNA, and protein labeling in the various brain regions examined, although to differing extents. This result is in agreement with morphological and biochemical investigations, indicating a selective vulnerability of the various brain regions during hypoxic conditions (6, 8, 21). Moreover, electrophysiological observations (10, 11) showed a reduced motor activity, an uncoordinated locomotion, and an inhability to perform fine movements in hypoxic conditions. All these modifications may be ascribed to cerebellar damage caused by the lack of oxygen.

The marked inhibition of DNA labeling in hypoxic conditions found in the present study may be related to the extensive neuronal necrosis observed by other authors (19, 35) in the brain during hypoxia. The impairment of DNA, RNA, and protein labeling may be related also to the breakdown of energy metabolism and to the decrease of endogenous levels of ATP and phosphocreatine found during oxygen deprivation (7, 26). The decreased ATP levels in hypoxic conditions might affect the active transport of labeled precursors into the cells, although active transport systems in brain were certainly demonstrated for amino acids (18) and not for nucleosides (16, 20, 22).

It is unlikely that the results obtained might be due to the changes in the specific radioactivity of the precursor pool. We obtained similar results in experiments performed in vivo and in vitro using different amounts of labeled precursors; moreover in the two experimental conditions the dilution of the radioactive precursor is certainly different. The decrease of RNA and protein labeling in hypoxic conditions, observed in the present study, is in agreement with several investigations performed in other laboratories with different experimental approaches and a variety of models of brain hypoxia (2, 6, 25, 32, 38–40). Yanagihara (38, 40), in isolated nuclei, with exogenously added nucleotides, found an inhibition of both RNA polymerase I and II activities during cerebral ischemia and anoxia. In isolated microsomes, incubated with exogenously added amino acids, he reported a marked decrease of protein synthesis. Moreover he found an alteration of polypeptide chain initiation in isolated polysomes (39). Therefore, the decrease of protein synthesis in hypoxic conditions is due to the changes of both transcriptional and posttranscriptional processes. Rossowska and Zalewska (30), in brain cortex of guinea pigs submitted to hypoxia, found that the concentration of amino acids in proteins of the microsomal fraction did not change significantly.

The decreased intensity of nucleic acid and protein labeling observed by us during hypoxic conditions, in total tissue, was reflected by similar changes in the various subcellular fractions examined, although a different vulnerability of the various subcellular fractions was found. Mitochondrial DNA labeling and microsomal protein labeling were the most affected processes by hypoxia. The higher vulnerability of the microsomal protein labeling agrees with other studies on cerebral anoxia (39), showing a decreased amino acid incorporation into proteins in the various subcellular fractions even after short periods of anoxia and higher sensitivity of the microsomal fraction. The impairment of microsomal protein labeling may be related to the disaggregation of polyribosomes reported in different pathophysiological conditions such as complete cerebral ischemia, anoxia, and hypoxia (39).

Concerning RNA labeling in the various subcellular fractions, it was affected to a different extent by the hypoxic treatment in the brain regions examined. Mitochondrial RNA labeling in the cerebellum was particularly affected. These results are in agreement with ultrastructural studies on nervous tissue, indicating a different vulnerability among cell organelles and an early mitochondrial damage during oxygen deprivation (4, 24). Electron microscopic observations showed mitochondrial swelling after only 3 min of oxygen deprivation (36). Also the specific activity of some mitochondrial enzymes (i.e., cytochrome oxidase and the enzymes of the tricarboxylic acid cycle) decreased rapidly during oxygen deprivation (17, 34, 37). Moreover, studies on oxidative metabolism during complete or incomplete cortical ischemia (29) indicated that the oxidative phosphorylation is uncoupled from the electron transport chain, suggesting that brain mitochondria are particularly sensitive to ischemia.

ACKNOWLEDGMENTS

This work was accomplished with financial support from C.N.R. (Italy). The skillful technical assistance of S. Reale and the secretarial help of D. Blarasin are greatly appreciated.

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