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Heat shock protein in mammalian brain and other organs after a physiologically relevant increase in body temperature induced by D-lysergic acid diethylamide

(hyperthermia/protein synthesis *in vivo*/cell-free translation)

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ABSTRACT A physiologically relevant increase in body temperature from 39.7 to 42.5°C, which was generated after the intravenous injection of D-lysergic acid diethylamide (LSD), caused the induction of synthesis of a 74,000-dalton heat shock protein in the brain, heart, and kidney of the young adult rabbit. A marked increase in the relative labeling of a 74,000-dalton protein was noted after analysis of both *in vivo* labeled proteins and cell-free translation products of isolated polysomes. A temporal decrease in the synthesis of this protein was noted as LSD-induced hyperthermia subsided. The 74,000-dalton protein, which is induced in various organs of the intact animal at a body temperature similar to that attained during fever reactions, may play a role in homeostatic control mechanisms.

Increase of ambient temperature has been reported to induce the synthesis of a specific set of heat shock proteins in a wide range of tissue culture systems and unicellular organisms (1–9). The exact number and molecular weight of the induced proteins is dependent on the particular system under investigation; however, the induction of several proteins in the molecular mass range of 68,000–74,000 daltons and 95,000 daltons is common to most systems (1, 3, 5).

Understanding the functional role that these proteins may play in mammalian cells is particularly important given the suggestion raised by Kelley and Schlesinger (3) that these proteins may be involved in the response of the body to fever reactions. An intriguing question that requires examination is whether heat shock proteins are induced within organs of the intact mammal during physiologically relevant increases in body temperature. In this report we demonstrate that an increase in body temperature similar to that attained during fever reactions induces the synthesis of a 74-kilodalton (kDal) protein in the brain, heart, and kidney of the young adult rabbit.

MATERIALS AND METHODS

Drug Administration. D-Lysergic acid diethylamide bitartrate (LSD), a product of Sandoz Pharmaceuticals, Switzerland, was obtained through the Department of National Health and Welfare, Ottawa, ON, Canada. The drug was dissolved in 0.9% NaCl for intravenous injection. Experiments were carried out on male New Zealand White rabbits (1.4–2.0 kg) kept on a 7 a.m.–7 p.m. light/dark cycle at 23°C and fed ad lib; LSD (100 μg/kg of body weight) was injected into a marginal ear vein between 9:00 and 10:30 a.m. Control animals received an appropriate volume of physiological saline. Colonic temperature was continuously monitored with a rectal thermistor probe (introduced 7–8 cm into the rectum) connected to a Y.S.I. model 47 telethermometer (accuracy, 0.015°C) (Yellow Springs Instrument). Only animals showing a normal body temperature of 39.7 ± 0.2°C prior to treatment were selected for experimentation. Animals that were injected with LSD had rectal temperatures of 42.5 ± 0.3°C 1 hr after drug administration. It has been shown (10) that rectal temperature in rabbits is an accurate representation of the body temperature of organs and that rectal temperature and organ temperature increase in parallel during hyperthermia.

***In Vivo* Labeling of Proteins.** Proteins in the cerebral hemisphere of the brain were labeled by injection of 0.5 mCi (1 Ci = 3.7 × 10^{10} becquerels) of [^{35}S]methionine (New England Nuclear) 20 min after the drug injection. The isotope was administered into the lateral ventricles of the brain through stereotaxically implanted stainless steel cannulae. The animals were pulsed for 1 hr and then dispatched by cervical dislocation. The cerebral hemispheres were dissected out and homogenized in 2 vol of 0.32 M sucrose/50 mM Hepes/KOH, pH 7.5/140 mM potassium acetate/5 mM magnesium acetate/2.5 mM dithiothreitol. The homogenate was centrifuged at 10,000 rpm for 10 min in a Sorvall SS34 rotor at 4°C to obtain a postmitochondrial supernatant.

Proteins in the kidney were labeled by intravenous injection into the marginal ear vein of 2.0 mCi of [^{35}S]methionine 45 min after drug injection. After a 45-min pulse, the animals were dispatched by cervical dislocation. The kidneys were homogenized in 5 vol of 0.25 M sucrose/50 mM Tris·HCl, pH 7.5/100 mM KCl/10 mM $MgCl_2$/5 mM dithiothreitol/200 μg of heparin sulfate per ml. A postmitochondrial supernatant was then prepared by centrifugation.

Isolation of Polysomes from Brain and Other Organs. Free polysomes were prepared as described (11) with the following modifications. The tissues were homogenized in 7 vol of homogenization buffer [0.25 M sucrose/50 mM Tris·HCl, pH 7.4/250 mM KCl/5 $MgCl_2$/2 mM dithiothreitol (homogenization buffer A) for the cerebral hemispheres, cerebellum, brain core, and kidney, and in 0.25 M sucrose/50 mM Tris·HCl, pH 7.4/250 mM KCl/2 mM $MgCl_2$/5 mM dithiothreitol/200 μg of heparin sulfate per ml (homogenization buffer B) for heart] with six passes of a motor-driven Teflon pestle (measured clearance, 0.15 mm) followed by two additional passes with a tighter Teflon pestle (measured clearance, 0.06 mm). The homogenate was centrifuged for 2 min at 2,000 rpm in a Beckman SW-27 rotor at 4°C, and then the speed was increased to 25,000 rpm for an additional 13 min to pellet rough microsomes. The resultant supernatant containing free polysomes was removed, adjusted to 1% Triton X-100, layered over 6 ml of homogenization buffer A containing 1.65 M sucrose, and centrifuged at 50,000 rpm

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Abbreviations: LSD, D-lysergic acid diethylamide; kDal, kilodaltons; RIPA, radioimmune precipitation assay.

for 3 hr in a Beckman 60 Ti rotor to pellet polysomes. Polysomes destined for cell-free translation were resuspended in 10 mM Hepes/KOH, pH 7.0/25 mM potassium acetate/5 mM magnesium acetate/1 mM dithiothreitol.

Cell-Free Translation of Polysomes. Reticulocyte lysate was prepared from phenylhydrazine-treated New Zealand White rabbits as described by Ranu and London (12). The lysate was treated with micrococcal nuclease and utilized in a cell-free translation system essentially as described by Pelham and Jackson (13). Each translation assay mixture (115 μl) contained 120 mM potassium acetate, 1 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 25 μM hemin, creatine kinase (50 μg/ml), 10 mM creatine phosphate, 50 μM each of 19 unlabeled amino acids except methionine, and 50–60 μCi of [^{35}S]methionine. Unless otherwise indicated, the mRNA-dependent reticulocyte lysate translation assay was programmed with nonsaturating amounts of polysomes (10 A_{260} units/ml). The reaction mixture was incubated at 30°C for 1 hr. To assay for acid-precipitable radioactivity, 5-μl aliquots were removed and diluted into 1 ml of ice-cold distilled H_2O. Next, 0.5 ml of 1 M KOH containing methionine (1 mg/ml) was added, and the reaction was incubated at 37°C for 20 min to release labeled amino acids bound to tRNA. The tubes were chilled, and 1 ml of ice-cold 25% trichloroacetic acid containing methionine (1 mg/ml) was added. The precipitates were collected on Whatman GF/C filters presoaked in 10% trichloroacetic acid containing methionine (1 mg/ml), and the filters were rinsed with the same solution, dried, and assayed for radioactivity in 10 ml of Triton X-100/toluene scintillation fluid (667 ml of toluene, 333 ml of Triton X-100, 5.0 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-bis[2(5-phenyloxazolyl)]benzene.

Analysis of Labeled Proteins by Gel Electrophoresis and Fluorography. Proteins were analyzed by two-dimensional gel electrophoresis (14). Aliquots containing equal amounts of acid-precipitable radioactivity from the cell-free protein synthesis system were solubilized in 10 vol of lysis buffer, [9.5 M urea/2% Nonidet P-40/2% Ampholines (Biorad; composed of 0.8% pH 4–6 Ampholines, 0.8% pH 6–8, and 0.4% pH 3–10)/5% 2-mercaptoethanol]. Aliquots of the postmitochondrial supernatant containing equal amounts of acid-precipitable radioactivity from the *in vivo* labeling experiments were first lyophilized to dryness and then solubilized in lysis buffer. Isoelectrofocusing in the first dimension was performed in 10-cm cylindrical gels containing 4% acrylamide, 9 M urea, and the 2% Ampholine carrier in the concentrations described above. As cathode and anode buffer, 20 mM NaOH (degassed) and 10 mM H_3PO_4 were used, respectively. Gels were run for 16 hr at 400 V, followed by 1 hr at 800 V. After the cylindrical gels were equilibrated for 30 min in $NaDodSO_4$ sample buffer (10% glycerol/5% 2-mercaptoethanol/2.3% $NaDodSO_4$/6.25 mM Tris·HCl, pH 6.8), they were fused with 0.75% agarose onto 7–17% acrylamide slab gels. Polyacrylamide slab gel electrophoresis in the presence of $NaDodSO_4$ was carried out with 5% stacking gels by utilizing the discontinuous buffer system of Laemmli (15). Gels were stained with Coomassie brilliant blue R and prepared for fluorography (16, 17).

Radioimmune Precipitation Assay (RIPA). Aliquots of postmitochondrial supernatant containing 10^5 cpm of *in vivo* labeled proteins from control and LSD-treated cerebral hemispheres were lyophilized, resuspended in 200 μl of RIPA buffer (1.0% Triton X-100/1.0% sodium deoxycholate/0.1% $NaDodSO_4$/0.15 M NaCl/0.05 M Tris·HCl, pH 7.2/1 mM phenylmethylsulfonyl fluoride) and mixed with 10 μl of antibody donated by M. Schlesinger, which was prepared against the chicken 70 kDal heat shock protein (18). The reaction was incubated for 30 min at room temperature. A 100-μl sample of a 10% solution of Formalin-treated staphylococcus protein A (19) was then added, and, after 20 min at room temperature, the mixture was pelleted in a Beckman microfuge. The pellet was washed three times with 0.4 ml of RIPA buffer and then twice with RIPA containing 1 M NaCl. The pellet was then resuspended in 50 μl of gel loading buffer (10% glycerol/2% $NaDodSO_4$/5% 2-mercaptoethanol/0.06 M Tris·HCl, pH 6.8/0.0001% bromophenol blue), boiled for 5 min, and pelleted. The supernatant was analyzed by $NaDodSO_4$ gel electrophoresis on 7–17% gradient slab gels.

RESULTS

Analysis of *in Vivo* Labeled Proteins After Drug-Induced Hyperthermia. Our previous results have demonstrated that the psychotropic drug LSD is a useful tool to probe translational mechanisms in the mammalian brain. Using *in vivo* and *in vitro* experimental techniques, we have shown that LSD binds to neurotransmitter receptors and rapidly induces a transient inhibition of protein synthesis in the rabbit brain through a lesion in reinitiation (20–24). Because we also have noted that LSD rapidly induces an increase in body temperature (i.e., hyperthermia) (20, 24), we were interested in investigating whether the drug induced the synthesis of heat shock proteins during the previously observed period of overall decrease in protein

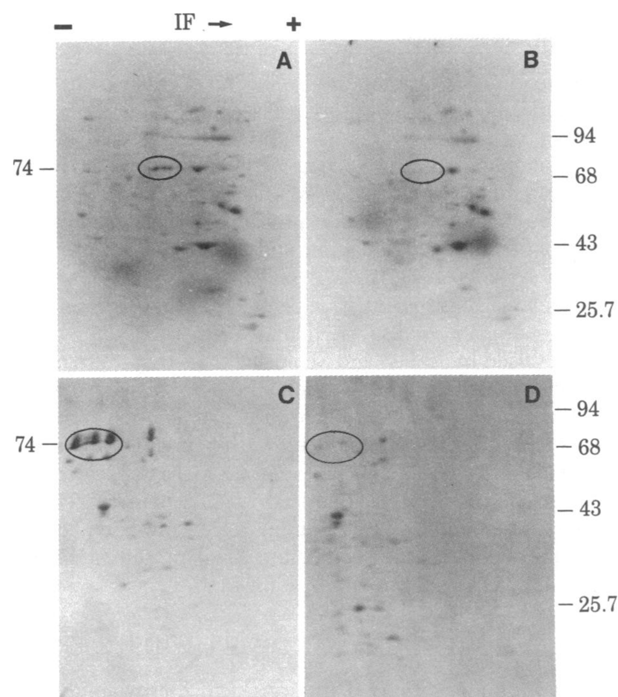

FIG. 1. Two-dimensional electrophoretic analysis of *in vivo* labeled proteins after drug-induced hyperthermia; sizes are shown in kDal. (*Left*) Drug-induced hyperthermia: cerebral hemispheres (*A*) and kidney (*C*). (*Right*) Saline controls: cerebral hemispheres (*B*) and kidney (*D*). Elevation of body temperature in rabbits was induced by the intravenous injection of LSD at 100 μg/kg of body weight. The body temperature of drug-treated animals increased from 39.7 ± 0.2 to 42.5 ± 0.3°C 1 hr after drug administration, whereas the temperature of saline-injected control animals remained unchanged. *In vivo* labeled proteins from kidney and cerebral hemispheres of the brain were obtained by pulse labeling with [^{35}S]methionine and preparation of a postmitochondrial supernatant fraction. Equal amounts of acid-precipitable radioactivity (50,000 cpm) from drug-treated and saline-treated control animals were analyzed by two-dimensional gel electrophoresis and fluorography. The position of the 74-kDal heat shock protein is encircled. IF, isoelectric focusing.

synthesis (for review of the mechanism of LSD-induced hyperthermia, see ref. 24).

As shown in Fig. 1, increase of body temperature from 39.7 ± 0.3 to 42.5 ± 0.3°C after intravenous LSD injection produced selective effects on brain protein synthesis. Analysis of *in vivo* labeled cerebral hemisphere proteins revealed an induction of synthesis of a 74-kDal protein relative to controls where body temperature remained at 39.7 ± 0.2°C (Fig. 1 *A* and *B*). Two-dimensional gel electrophoresis resolved this protein into three spots. Induction of the 74-kDal protein appeared to be correlated with drug-induced hyperthermia (i.e., increase of body temperature) because prevention of hyperthermia by injection of the drug into rabbits maintained in a cold room at 4°C eliminated the phenomenon (data identical to control shown in Fig. 1*B*); however, behavioral effects of the drug were still apparent (20). Under these conditions body temperature remained at 39.7°C. Increase of body temperature to 42.5°C by means other than LSD (i.e., placement of rabbits in an incubator at 37°C) also induced the synthesis of the 74-kDal protein (data identical to Fig. 1*A*). Body temperature increased under these conditions because the animals were unable to lose body heat through their ears due to the elevated ambient temperature. After drug-induced hyperthermia, increased synthesis of the 74-kDal protein also was observed when *in vivo* labeled proteins from kidney were examined (Fig. 1 *C* and *D*). In kidney, labeling of the 74-kDal protein was detected in trace amounts in control animals (Fig. 1*D*).

To determine whether the 74-kDal protein induced in the rabbit brain after increase of body temperature was similar to the heat shock protein induced in other systems, *in vivo* labeled proteins were treated with antibody prepared against the 70-kDal chicken heat shock protein (18). The resultant precipitate was then analyzed by $NaDodSO_4$ gel electrophoresis (Fig. 2). Increased labeling of the 74-kDal protein was apparent in the total spectrum of *in vivo* labeled proteins from the LSD-treated cerebral hemispheres (Fig. 2, lane 3) as compared to those from the control cerebral hemispheres (lane 1). Antibody to the 70-kDal chicken heat shock protein precipitated the 74-kDal protein from LSD-treated cerebral hemispheres (Fig. 2, lane 4) and to a lesser extent from the control cerebral hemispheres (lane 2). In addition there was some crossreaction with a 95-kDal protein in both the control and LSD-treated cerebral hemispheres.

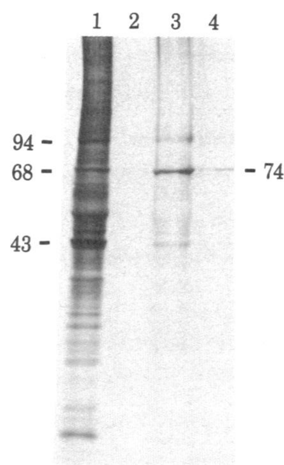

FIG. 2. Immunoprecipitation of *in vivo* labeled brain proteins (sizes shown in kDal) from control and LSD-treated rabbits by antibody to the 70-kDal chicken heat shock protein. Lanes: 1, total *in vivo* labeled proteins from control cerebral hemispheres; 2, *in vivo* labeled proteins from control cerebral hemispheres immunoprecipitated with antibody to the 70-kDal chicken heat shock protein; 3, total *in vivo* labeled proteins from LSD-treated cerebral hemispheres; 4, *in vivo* labeled proteins from LSD-treated cerebral hemispheres immunoprecipitated with antibody to the 70-kDal chicken heat shock protein. Equal amounts of acid-precipitable radioactivity (100,000 cpm) of $[^{35}S]$methionine-labeled brain proteins from the postmitochondrial supernatant of control and LSD-treated cerebral hemispheres were immunoprecipitated. Proteins were resolved by $NaDodSO_4$ gel electrophoresis on a 7–17% gradient slab gel and fluorography.

Cell-Free Translation of Purified Polysomes After Drug-Induced Hyperthermia. To investigate whether the change in synthesis of the 74-kDal protein shown in Fig. 1 was due to an increase in mRNA in polysomes or a change in protein modification, purified polysomes were translated in a reticulocyte cell-free system. Increase of body temperature from 39.7 ± 0.2 to 42.5 ± 0.3°C after drug injection resulted in a selective increase in the labeling of a 74-kDal protein in polysomes from various organs of the adult rabbit. Analysis of the translation products of polysomes isolated from two brain regions [i.e., cerebral hemispheres and brain core (Fig. 3 *A*–*D*)] and from heart and kidney (Fig. 4 *A*–*D*) revealed selective increases in labeling of a 74-kDal protein relative to controls whose body temperature remained at 39.7 ± 0.2°C. When increase of body temperature was prevented by injection of LSD to rabbits maintained at 4°C, increased labeling of the 74-kDal protein was not observed (data similar to controls shown in Figs. 3 and 4).

The 74-kDal protein may be a normal cellular constituent because its presence is detectable at low levels in the translation products of polysomes isolated from the brain core and heart

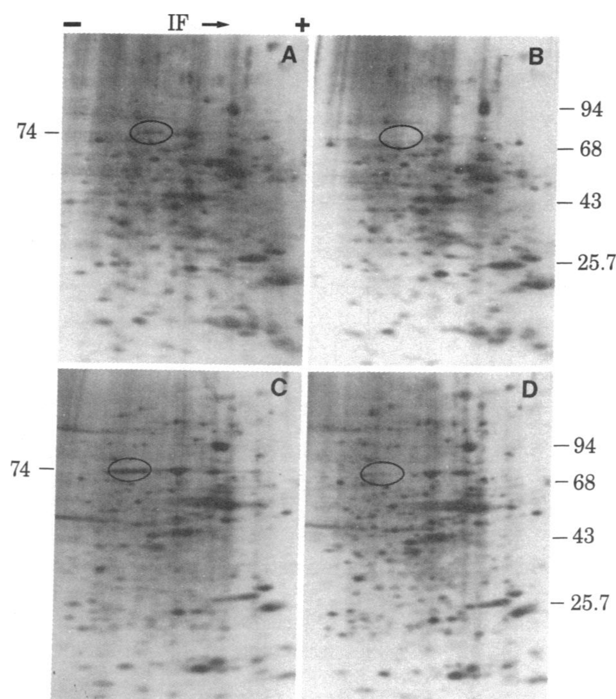

FIG. 3. Cell-free translation of brain polysomes after drug-induced hyperthermia. The position of the 74-kDal heat shock protein is encircled. (*Left*) Drug-induced hyperthermia: cerebral hemispheres (*A*) and brain core (*C*). (*Right*) Saline controls: cerebral hemispheres (*B*) and brain core (*D*). A mRNA-dependent reticulocyte lysate was programmed with free polysomes isolated from two regions of the rabbit brain 1 hr after the intravenous injection of LSD, which increased body temperature from 39.7 to 42.5°C. Equal amounts of acid-precipitable radioactivity (200,000 cpm) from drug-treated and saline-injected control animals were analyzed by two-dimensional gel electrophoresis and fluorography. Sizes are shown in kDal. IF, isoelectric focusing.

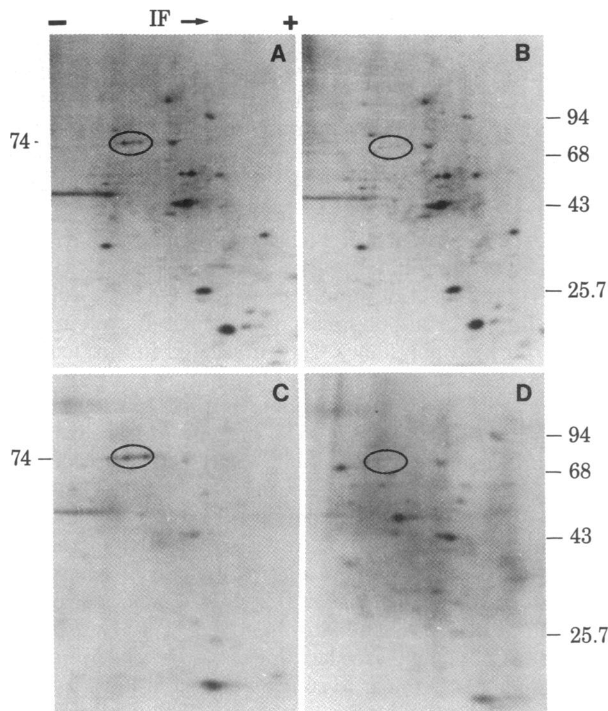

FIG. 4. Cell-free translation of heart and kidney polysomes. The 74-kDal heat shock protein is encircled. (*Left*) Drug-induced hyperthermia: heart (*A*) and kidney (*C*). (*Right*) Saline controls: heart (*B*) and kidney (*D*). After drug-induced hyperthermia as described in Fig. 2, polysomes were isolated from heart and kidney and translated in a reticulocyte cell-free system. The labeled translation products (sizes shown in kDal) were then analyzed by electrophoresis and fluorography. IF, isoelectric focusing.

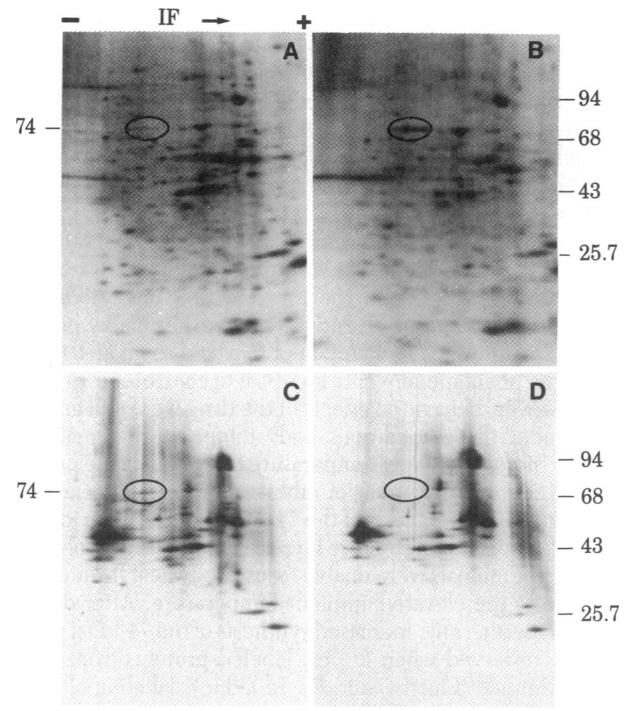

FIG. 5. Temporal changes in synthesis of the 74-kDal protein by cerebellar polysomes. Polysomes were isolated from the cerebellum 1, 4, and 8 hr after drug administration, translated in a reticulocyte cell-free system and the labeled products analyzed as in Fig. 3. Whereas body temperature increased to 42.5°C 1 hr after drug administration, by 4 hr it had decreased to 41.0°C, and by 8 hr recovery to the normal body temperature of 39.7°C had occurred. The position of the 74-kDal heat shock protein is encircled in the saline control (*A*) and in samples isolated 1 hr (*B*), 4 hr (*C*), and 8 hr (*D*) after LSD administration. Sizes are shown in kDal. IF, isoelectric focusing.

of control animals. The relative synthesis of this protein was greatly enhanced after drug-induced hyperthermia. The results shown in Figs. 3 and 4 suggest that the increase in the 74-kDal protein is due to an increase in mRNA in polysomes rather than a change in protein modification.

Temporal Changes in the Synthesis of the 74-kDal Protein. The results shown in Figs. 1, 3, and 4 demonstrate a relative increase in the synthesis of a 74-kDal protein in various organs of the adult rabbit, 1–1.5 hr after the injection of LSD. A maximal body temperature of 42.5 ± 0.3°C was attained 1 hr after injection of LSD at 100 μg/kg of body weight. By 4 hr, body temperature had decreased to 41.0 ± 0.3°C; by 8 hr, recovery to the normal body temperature of 39.7 ± 0.2°C had occurred.

Polysomes were isolated from the cerebellum of the brain, 1, 4, and 8 hr after drug administration and translated in the reticulocyte cell-free system. At 4 hr (Fig. 5*C*), synthesis of the 74-kDal protein was increased relative to controls (Fig. 5*A*); however, the effect was less pronounced compared to that observed at 1 hr (Fig. 5*B*). By 8 hr (Fig. 5*D*), the synthesis of the 74-kDal protein was not detectable. Similar results were obtained at 4 and 8 hr with polysomes purified from the cerebral hemispheres and brain core and from heart and kidney (data not shown). These results suggest that the selective increase in the synthesis of the 74-kDal protein is transient and related to the increase in body temperature.

DISCUSSION

The data suggest that increase of body temperature from 39.7 to 42.5°C after LSD administration results in a selective increase in the synthesis of a 74-kDal protein in the brain, heart, and kidney of the young adult rabbit. This protein is similar in molecular mass to one of the major heat shock proteins previously reported to be induced in a wide range of tissue culture systems and unicellular organisms after increase of ambient temperature (1–9). The 74-kDal protein induced in brain is precipitated by antibodies prepared against chicken 70-kDal heat shock protein. The time course of synthesis of the 74-kDal protein appears to parallel the transient increase in body temperature that is induced by the intravenous injection of LSD. It has been suggested that heat shock proteins may play an important role in the response of the body to fever reactions (3); however, studies on these proteins have focused generally on single-cell systems rather than on organ systems in intact animals. The present results suggest that a physiologically relevant increase in body temperature of 3°C, similar to that attained in fever reactions (24), results in a marked increase in synthesis of a 74-kDal protein in all organs of the rabbit that were examined.

In tissue culture systems and unicellular organisms, increase of ambient temperature induces the synthesis of a set of heat shock proteins (1–9), whereas, in the intact-organ situation reported in this study, a marked increase in synthesis of only a 74-kDal protein is observed after increase of body temperature. Because mammalian cell lines appear to have genes coding for a set of heat shock proteins (3, 25, 26), it is of interest that in intact organs only one of the major heat shock proteins is increased in synthesis. It has been reported that the pattern and extent of induction of heat shock proteins in *Drosophila* is dependent on the magnitude and rate of the increase in ambient temperature (27). The rate of increase in body temperature after LSD administration is slow in comparison to the increase of

ambient temperature in many tissue culture experiments (28). It would appear that constraints exist on the induction of the full set of heat shock proteins at physiologically relevant temperatures in organs of the intact animal. This may suggest an adaptation of the 74-kDal heat shock protein to a functional role in the cellular reaction of intact organ systems to thermal stress conditions.

In this report, the hyperthermia-induced increase in synthesis of the 74-kDal protein was demonstrated by two methods—i.e., *in vivo* labeling of proteins and translation of purified polysomes in a reticulocyte cell-free system. The increased synthesis of the 74-kDal protein, which was observed in the polysome translation experiments, suggests that the change is due to an increase in the relative abundance in polysomes of mRNA coding for this protein and not to change in protein modification. Whether this change in polysomal mRNA reflects movement of newly synthesized mRNA from the nucleus or movement of preexisting mRNA into polysomes is not known at present. In tissue culture systems (1, 29), increase of ambient temperature induces the transcription of genes coding for heat shock proteins. Recent *in vitro* experiments with the rabbit retina suggest that the temperature-induced increase in synthesis of the 74-kDal protein is dependent on RNA synthesis (unpublished data).

The 74-kDal protein appears to be a normal cellular constituent in various organs of the rabbit because its presence is detectable at low levels among the *in vivo* labeled proteins and in the translation products of isolated polysomes. This is in agreement with studies in other systems that have reported the presence of low levels of heat shock proteins prior to increase of ambient temperature (3, 25, 30). After increase of body temperature in the rabbit and induction of the 74-kDal protein, a full spectrum of other cellular proteins appears to be synthesized, although at a reduced rate. In avian and mammalian tissue culture cells, a full range of cellular proteins also appears to be synthesized (3); however, in other systems such as *Drosophila*, primarily heat shock proteins appear to be synthesized after an increase of temperature (31).

Other factors have been reported to induce proteins similar in molecular weight to heat shock proteins—i.e., amino acid analogs (1, 3, 26), heavy metal ions (32), sulfhydryl reagents (32), and chelating agents (33, 34). A trauma-induced protein of 71-kDal, which shows tissue-specific charged variants, has been reported in incubated organ slices and in tissues of the anesthetized rat (26, 35–37). It is possible that a common set of proteins is induced by diverse treatments as a general cellular response to stress.

The present report demonstrates that a 74-kDal protein is markedly increased in synthesis in various organs of an intact mammal in response to a physiologically relevant increase in body temperature. It is possible that this protein may be involved in homeostatic control mechanisms during thermal stress such as is attained during fever reactions.

Note Added in Proof. We recently have found that increases in body temperature also induce the synthesis of a 74-kDal protein in the rabbit retina (38), the cerebral microvascular system (39), and the fetal and neonatal brain (40).

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