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Detection of HSP 72 synthesis after acoustic overstimulation in rat cochlea

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The purpose of this study was to determine if high intensity acoustic stimulation would induce HSP 72 in rat cochlea. The animals were exposed to 110 dB SPL broad band noise for 1.5 h and sacrificed 4, 6 and 8 h after stimulation. Immunocytochemistry and western blotting were used to detect the expression of HSP 72 in the cochlear tissues. Western blots showed an intense 72 kD band in the noise exposed animals compared to a very light band in non-stimulated control animals. Immunocytochemical results in the cochlea revealed noise induced HSP 72 immunoreactive staining of outer hair cells. Only a few immunoreactive stained inner hair cells were seen and spiral ganglion cells were not stained. These results indicate that acoustic overstimulation can induce the expression of HSP 72 in outer hair cells of the rat cochlea. HSP 72 may serve as a marker for cellular stress and potential damage and may be involved in protection from insult.

HSP 72; Noise; Cochlea; Hair cell; Immunoblotting; Immunocytochemistry; Rat

Introduction

Heat shock proteins (HSPs) are induced after the exposure of cells to various metabolic and environmental stresses (Linquist and Craig, 1988). Although first described after hyperthermic stimuli (Tissieres et al., 1974), HSPs are now commonly referred to as stress response proteins because of their expression after various physiological stresses as well as heat shock (Linquist and Craig, 1988). The stress response proteins are thought to have a role of cellular protection perhaps by means of solubilizing and refolding denatured or misfolded proteins which have resulted from various cellular stresses (Linquist, 1986; Welch, 1990). Among several proteins of the HSP family, the best studied stress response proteins are those in the 70 kD family. In mammalian cells there are two major members of the 70 kD family: an abundant, constitutive 73 kD protein and a highly stress inducible 72 kD protein (Welch and Suhan, 1986).

A number of environmental and physiological stresses, e.g. noise, ototoxic drugs, hypoxia are known to cause transient or permanent pathological damage to cochlea (for review: Lim, 1986; Libermann, 1990). HSPs are believed to be induced by moderate stresses in order to protect the cells from even more severe stresses (Lindquist and Craig, 1988) and it can be hypothesized that HSPs may have some responsibilities in protective mechanism of hair cells against various kinds of insults. In the peripheral auditory system several investigators have demonstrated the induction of HSP 72 with hyperthermia in the rat cochlea (Dechesne et al., 1992) and guinea pig cochlea (Thompson et al., 1992), as well as with transient ischemia in the rat cochlea (Myers et al., 1992).

We investigated whether another more common stress to the auditory system, acoustic overstimulation, could induce HSP 72 expression in cochlear tissue. Immunohistochemical and western blot analyses were used to detect the HSP 72 expression in rat cochlea.

Materials and Methods

Animals and noise exposure

Forty-four male Sprague-Dawley rats (200–250 g) were used in this study. Thirty-two rats (8 rats each for 4, 6 and 8 h after noise exposure and non-stimulated control rats) were used for immunocytochemistry and 12 rats (6 noise exposed and 6 non-stimulated control) were used for western blot analysis. The experimental animals were exposed to 110 dB SPL broad band noises with pulses (5/s, 50% duty cycle) for 1.5 h in a soundproof booth. After the termination of acoustic overstimulation, the animals were placed in a quiet sound proof booth until the time of sacrifice. The control animals did not receive any stimulation and

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were placed in a quiet sound proof booth until the time of sacrifice. The care and use of animals reported on in this study were approved and supervised by the University of Michigan Unit on Laboratory Animal Medicine under grants from the Deafness Research Foundation and NIA grant AG05885 'Mechanisms of Age-Related Auditory Sensory Deficits'.

Immunocytochemistry

Four, six and eight hours after cessation of stimulation, subjects were deeply anesthetized with 17.5% chloral hydrate (0.2 ml/100 gm) and intracardially perfused with 4% paraformaldehyde. Temporal bones were removed and cochleae received intrascalar fixation with same fixative. Cochleae were then microdissected for surface preparation or decalcified with 3% EDTA at 4°C for 3–5 days and rapidly frozen with liquid nitrogen for cryostat section. Frozen cochleae were sectioned (15 mm) at −20°C, and thaw-mounted on gelatin coated slide. Immunoperoxidase and immunofluorescent immunocytochemistry were carried out on both surface preparations and cryostat sections of cochlea using monoclonal antibodies against HSP 72 (Amersham, Arlington Heights, IL). The Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and rhodamine conjugated antimouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were used for detection of primary antibody.

Electrophoresis and western blot

All reagents were obtained from Sigma Chemical Co., St. Louis, MO. unless otherwise noted. Six hours after noise exposure, the animals were deeply anesthetized with intraperitoneal injection of 17.5% chloral hydrate (0.2 ml/100 gm) and sacrificed by decapitation. Temporal bones were rapidly dissected in Hank's balanced salt solution (Gibco, Gaithersburg, MD) without sodium bicarbonate and phenol red, with added 5 mM sodium *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4 at 4°C. Cochlear tissues were divided into two parts: sensoriepithelium with lateral wall (spiral ligament and stria vascularis) and modiolus including spiral ganglion and auditory nerve. The tissues were then homogenized in homogenization buffer (10 mM HEPES, pH 7.4, 0.2 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetra-acetic acid (EGTA) with 200 mM phenylmethylsulfonylfluoride, 2 mM pepstatin A, and 2 mM leupeptin as protease inhibitors) and centrifuged for 1 minute at 14,000 × *g*. The supernatents were used for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Tissues from control animals were obtained in the same manner as noise exposed animals. Protein concentration was determined using dye binding protein assay with bovine serum albumin as a relative protein standard (Bradford, 1976). Gel samples were made using 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromophenol blue and boiling for 5 min. Thirty mg of protein was loaded into each lane and the slab minigels (3–12% acrylamide gradients from Jule Inc., New Haven, CT) were electrophoresed at 35 mA constant current for 3 h (Laemmli, 1970). The separated proteins were electrophoretically transferred to nitrocellulose membrane (Millipore Corp., Bedford, MA) at 100 mA constant current for 16 h according to the method of Towbin et al. (1979). The blots were treated with Blotto (5% dried milk and 0.2% Tween 20 in 20 mM Tris-HCl, 500 mM NaCl [TBS], pH 7.5) for 1 hour at room temperature to block nonspecific binding and then incubated with mouse monoclonal anti-HSP 72 antibody diluted 1:1000 in Blotto, overnight at 4°C. After washing with several changes of Blotto, the blots were incubated with biotinylated horse antimouse IgG (Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:400 in Blotto for 1 hour at room temperature

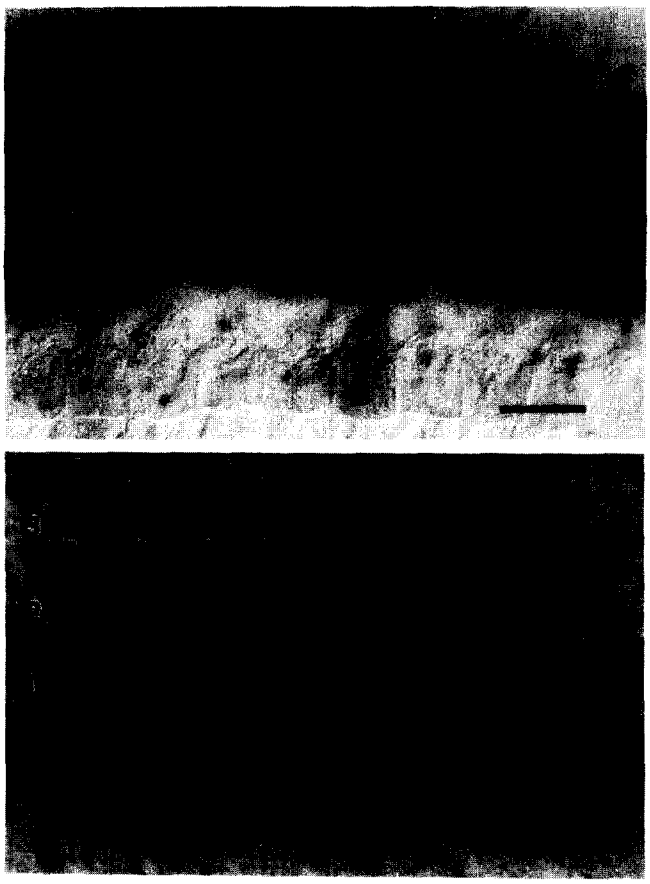

Fig. 1. (a): Surface preparation from the second turn of rat cochlea stimulated with 110 dB SPL of broad band noise immunoreacted with antibody to HSP 72. HSP 72 immunoreactivity is seen in all three rows (1–3) of outer hair cells. (b): Surface preparation from the second turn of non-stimulated rat cochlea immunoreacted with antibody to HSP 72. No HSP 72 immunoreactive staining is seen. OHC = outer hair cells, scale bar = 10 μm.

and then incubated with an avidin-biotinylated horse radish peroxidase (HRP) complex (Vector Laboratories, Inc.) for 45 min and then rinsed with a change of fresh Blotto, and two changes of TBS. The bound HRP was detected with substrate 4-chloro-1-naphthol by using 30 mg/10 ml of methanol, 50 ml of TBS and 165 ml of 30% hydrogen peroxidase as the chromogen.

Results

Immunocytochemistry

Immunoperoxidase and immunofluorescent techniques showed the same results. In control animals (without noise exposure), there was no specific immunostaining in either surface preparations (Fig. 1b) or cryostat sections. In noise exposed animals, HSP 72 immunoreactive staining was observed in all three rows of outer hair cells (Fig. 1a, 2a) and stria vascularis (Fig. 2c). Maximal immunoreactive staining was observed 6 h after noise exposure. Fewer outer hair cells were immunostained and staining was lighter in 4 and 8 h animals. Immunostaining was mainly in the cytoplasm of outer hair cells without nuclear staining (Fig. 2b). Only a few immunoreactive stained inner hair cells were seen and supporting cells and spiral ganglion cells were not stained. While immunostaining of outer hair cells was seen in all rows and turns of the cochlear spiral, unlabelled outer hair cells were more evident (25–50%) in the first turn, while almost all outer hair cells were labelled in more apical turns.

Western blot analysis

HSP 72 was detected in noise exposed rat cochlear tissues by immunostaining of blots using the same antibody as in immunocytochemistry. Intense bands

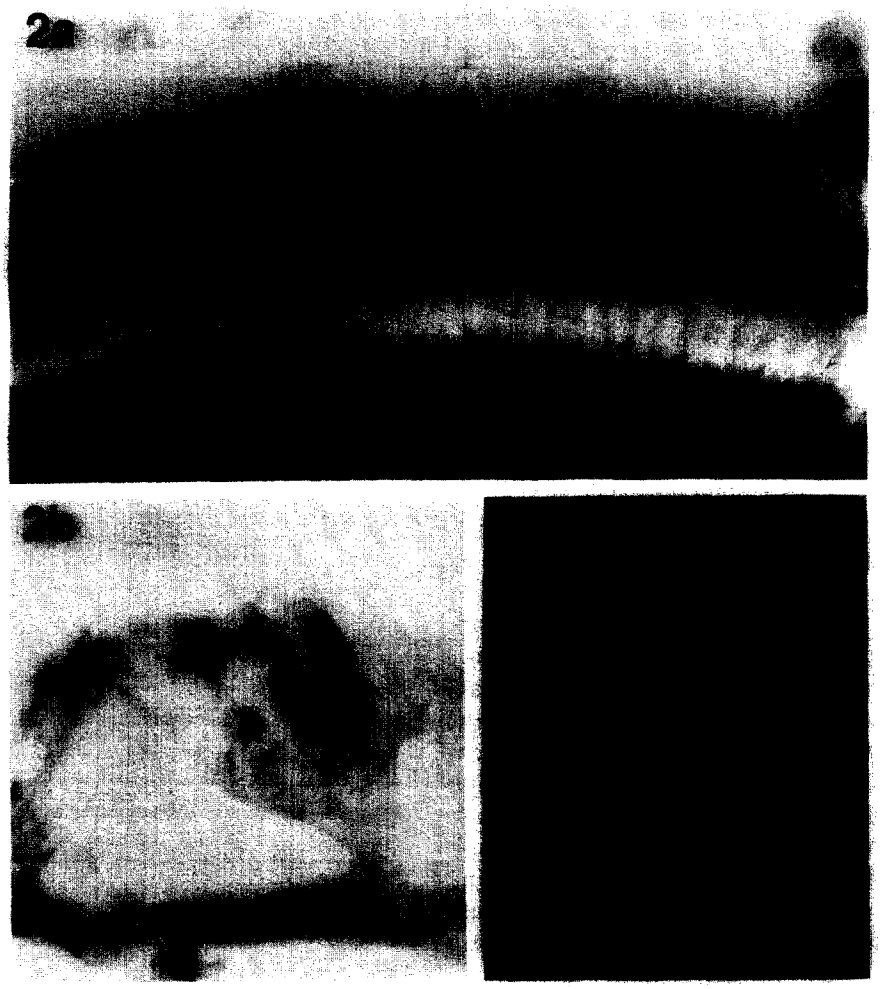

Fig. 2. Surface preparations and sections of rat cochlea stimulated with 110 dB SPL of broad band noise, sacrificed 6 h after noise exposure and immunoreacted with antibody to HSP 72. (a): Surface preparation from the second turn of rat cochlea with immunoperoxidase staining. Three rows of outer hair cells are intensely immunostained for HSP 72. OHC = outer hair cell, scale bar = 10 μm. (b): Cryostat section from the second turn of rat cochlea with immunoperoxidase staining. HSP 72 immunostaining of outer hair cells is seen, mainly in their cytoplasm, without nuclear staining. Scale bar = 10 μm. (c): Cryostat section from the second turn of rat cochlea with immunofluorescent staining. Intense HSP 72 immunoreactive labeling is seen in the stria vascularis. SV = stria vascularis, scale bar = 10 μm.

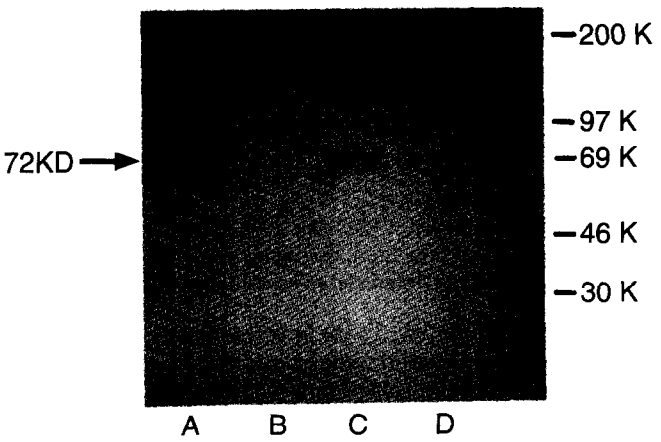

Fig. 3. Western blot analysis of sensoriepithelium with lateral wall (spiral ligament and stria vascularis) and modiolus from noise exposed (110 dB SPL) and non-stimulated rat cochlea using monoclonal antibody to HSP 72. Intense bands at M.W. 72 kD are seen in noise exposed sensoriepithelium (A) and modiolus (C) compared to very light, weaker bands in non-stimulated cochleae (B, D). A: noise exposed sensoriepithelium, B: non-stimulated control sensoriepithelium, C: noise exposed modiolus, D: non-stimulated control modiolus.

were found at M.W. 72 kD in tissues from sensoriepithelium with lateral wall of cochlea and modiolus including spiral ganglion and auditory nerve. A less intense, heavier molecular weight band was also seen, perhaps representing a dimer of HSP 72. In control rats, very light bands, much weaker than in noise exposed rats, were seen (Fig. 3).

Discussion

There is now considerable evidence that HSPs are induced in mammalian cells after exposure to various kinds of metabolic and environmental stresses. A number of studies using mammalian brain have demonstrated an increased synthesis of HSPs after a variety of stresses including hyperthermia (Sprang and Brown, 1987; Masing and Brown, 1989; Brown and Rush, 1990; Marini et al., 1990; Nowak er al, 1990) and transient ischemia (Daniel et al, 1986; Vass et al, 1988; Nowak et al., 1990). In the auditory system, induction of HSPs in cochlea has been assessed in rats and guinea pigs by several investigators. While the normal, non-stressed guinea pig cochlea has a high level of expression of HSP 72 in many regions (Neely et al., 1991), a heat induced increased expression of HSP 72 immunoreactivity in interdental cells of the spiral limbus was observed by Thompson et al. (1992). Dechesne et al. (1992) and Myers et al. (1992) found no HSP 72 expression in the normal, non-stressed, rat cochleae and induced expression using heat and hypoxia respectively. We stimulated rats with intense noise, a stress that is more specific and common to the cochlea. Although we did not follow up the long term fate of HSP 72 expression, HSP 72 immunoreactivity was detected at 4, 6 and 8 h. Maximal expression was 6 h after cessation of stimulation which is similar to other studies (Dechesne et al., 1992; Thompson et al., 1992) in rats and guinea pigs. Our immunocytochemical data showed HSP 72 immunoreactivity in three rows of outer hair cells, only rarely in inner hair cells and not in spiral ganglion cells. These results are comparable to the preliminary results of Myers et al. (1991) that revealed HSP 72 expression in outer hair cells following transient ischemia. However, these results are different from the Dechesne et al. (1992) who demonstrated heat induced HSP 72 expression in spiral ganglion cells, spiral ligament and stria vascularis but not in the organ of Corti with both western blot and immunocytochemical analyses. They suggested the lack of expression of HSP 72 in organ of Corti might be due to an insufficient amount of protein applied on the gel, however it is also possible that hypoxia and acoustic overstimulation are more effective at inducing HSP 72 in outer hair cells than hyperthermia.

The detection of HSP 72 immunostaining in outer hair cells and not inner hair cells may be due to several factors. Outer hair cells are generally more susceptible to acoustic trauma than inner hair cell (Liberman and Beil, 1979; Erlandsson et al, 1980; Robertson and Johnstone, 1980; Libermann and Mulroy, 1982). Outer hair cells may be more stressed than inner hair cells and therefore more likely to express HSP 72. It would be interesting to use acoustic overstimulation paradigms reported to have more effect on inner hair cells (Engström and Borg, 1981; Engström et al, 1983) to see if inner hair cells express HSP 72 under these conditions. On the other hand outer hair cells may have a lower threshold for induction of HSP 72. It will also be interesting to examine the localization of distribution of HSP 72 in hair cells after noise exposure with different intensity and frequency.

The absence of noise-induced immunostaining of spiral ganglion cells despite western blot results showing a noise-induced 72 kD band is interesting. This suggests that western blotting on unfixed tissue may be more sensitive than immunocytochemistry on fixed tissue. It also suggests that we must be careful in interpreting immunocytochemical 'negatives'.

Immunolabeling of HSP 72 was also seen in stria vascularis. This finding is in agreement with previous studies using hyperthermia and ischemic stresses (Dechesne et al., 1992; Myers et al., 1992). As in hyperthermic and ischemic stresses, localized capillary vasoconstriction as a results of noise overstimulation, which causes lower oxygen tension and nutrient supply to cochlea (Hawkins, 1971; Quirk et al., 1991), also may serve a condition to induce HSPs expression in stria vascularis and microvasculature.

The exact function of HSP 72 in auditory system is unclear, but it can be postulated that presence of HSP 72 in outer hair cells after noise overstimulation may play a important role to protect cells from further damage. Further studies will examine expression of HSPs under more specific stimulus condition and examine the correlation to hair cell loss.

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References

Blake M.J., Nowak T.S. Jr. and Holbrook, N.J. (1990) In vivo hyperthermia induces expression of HSP70 mRNA in brain regions controlling the neuroendocrine response to stress. Mol. Brain Res. 8, 89–92.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitiation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem. 72, 248–254.

Brown, I.R. and Rush, S.J. (1990) Expression of heat shock genes (hsp70) in the mammalian brain: Distinguishing constitutively expressed and hyperthermia-induced mRNA species. J. Neurosci. Res. 35, 14–19.

Daniel, G.A., Kiessling, M., Jacewicz, M. and Pulsinelli, W.A. (1986) Synthesis of heat shock proteins in rat brain cortex after transient ischemia. J. Cereb. Blood Flow Metab. 6, 505–510.

Dechesne, C.J., Kim, H.N., Nowak Jr, T.S. and Wenthold, R.J. (1992) Expression of heat shock protein, HSP 72, in guinea pig and rat cochlea after hyperthermia: Immunochemical and in situ hybridization analysis. Hear. Res. 59, 195–204.

Engström, B. and Borg, E. (1981) Lesions to cochlear inner hair cells induced by noise. Arch. Otorhinolaryngol. 230, 279–284.

Engström, B., Flock, Å. and Borg, E. (1983) Ultrastructural studies of stereocilia in noise-exposed rabbits. Hear. Res. 12, 251–264.

Erlandsson, B., Hakanson, H., Ivarsson, A., Nilsson, P. and Wersall, J. (1980) Hair cell damage in the guinea pig due to different kinds of noise. Acta Otolaryngol. Suppl. 367, 1–43.

Hawkins, J.E., Jr (1971) The role of vasoconstriction in noise-induced hearing loss. Ann. Rhinol. Laryngol. 80, 903.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Libermann, M.C. and Beil, D.G. (1979) Hair cell condition and auditory nerve response in normal and noise-damaged cochleas. Acta Otolaryngol. 88, 161–176.

Libermann, M.C. (1990) Quantitative assessment of inner ear pathology following ototoxic drugs or acoustic trauma. Toxicol. Pathol. 18, 138–149.

Libermann, M.C. and Mulroy, M.J. (1982) Acute and chronic effects of acoustic trauma: Cochlea pathology and auditory nerve pathophysiology. In: R.P. Hamernik, D.Henderson and R. Salvi (Eds.), New perspectives on noise-induced hearing loss. Raven press, NY, pp. 105.

Lim, D.J. (1986) Effects of noise and ototoxic drugs at the cellular level in the cochlea. Am. J. Otolaryngol. 7, 73–99.

Lindquist, S. (1986) The heat-shock response. Annu. Rev. Biochem. 55, 1151–1191.

Lindquist,S. and Craig, E.A. (1988) The heat shock protein. Annu. Rev. Genet. 22, 631–677.

Manzerra, P. and Brown, I.R. (1990) Time course of induction of a heat shock gene (hsp 72) in the rabbit cerebellum after LSD in vivo: Involvment of drug-induced hyperthermia. Neurochem. Res. 15, 53–59.

Marini, A.M., Kozuka, M., Lipsky, R.H. and Nowak, T.S. Jr. (1990) 70-kilodalton heat shock protein induction in cerebellar astrocytes and cerebellar granule cells in vitro: comparison with immunocytochemical localization after hyperthermia in vivo. J. Neurochem. 54, 1509–1516.

Masing, T.E. and Brown, I.R. (1989) Cellular localization of heat shock gene expression in rabbit cerebellum by in situ hybridization with plastic embedded tissue. Neurochem. Res. 14, 725–731.

Myers, M.W., Quirk, W.S., Rizk, S.S., Miller, J.M. and Altschuler, R.A. (1992) Expression of the major mammalian stress protein in the rat cochlea following transient ischemia. Laryngoscope 102, 981–987.

Neely, J.G., Thompson, A.M. and Gower, D.J. (1991) Detection and localization of heat shock protein in the normal guinea pig cochlea. Hear. Res. 52, 403–406.

Nowak, T.S. Jr. (1985) Synthesis of a stress protein following transient ischemia in the gerbil. J. Neurochem. 45, 1635–1641.

Nowak, T.S. Jr. (1991) Localization of 70KDa stress protein mRNA induction in gerbil brain after ischemia. J. Cereb. Blood Flow Metab. 11, 432–439.

Quirk, W.S., Shapiro, B.D., Miller, J.M. and Nuttall, A.L. (1991) Noise induced changes in red blood cell velocity in lateral wall vessels of the rat cochlea. Hear. Res. 52, 217–224.

Robertson, D. and Johnstone, B.M. (1980) Acoustic trauma in the guinea pig cochlea: Early changes in ultrastructural and neural thresholds. Hear. Res. 3, 167–179.

Schlesinger, M.J. (1990) Heat shock proteins. J. Biol. Chem. 265, 21, 12111–12114.

Thompson, A.M. and Neely, J.G. (1992) Induction of heat shock protein in interdental cells by hyperthermia. Otolaryngol. Head & Neck Surg. vol 107, No. 6: 769–774.

Tissieres A., Mitchell, H.K. and Tracy, U. (1974) Protein synthesis in salivary glands of Drosophilic melanogaster: relationship to chromosome puffs. J. Mol. Biol. 84, 389–398.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. Proc. Natl. Acad. Sci. USA. 76, 4350–4356

Vass, K., Welch, W.J. and Nowak, T.S. Jr. (1988) Localization of 70-KDa stress protein induction in gerbil brain after ischemia. Acta neuropathol. 77, 128–135.

Welch W.J. (1990) The mammalian stress response: cell physiology and biochemistry of stress proteins. In: R.I. Morimoto, A. Tissieres and C. Georgopoulos (Eds.), Stress proteins in biology and medicine. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 223–278.

Welch, W.J. and Suhan, J.P. (1986) Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell Biol. 103, 2035–2052.