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Gene expression and induced ischemic tolerance following brief insults

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Abstract. Striking changes in gene expression occur after transient ischemic insults, including the induction of heat shock proteins that are believed to play a central role in cellular defense mechanisms, and proto-oncogene (immediate-early gene) transcription factors that regulate the expression of diverse target genes. Such effects potentially contribute to a wide range of pathophysiological responses. A number of studies have characterized models of induced ischemic tolerance in which brief priming insults are demonstrated to result in reduced vulnerability to subsequent challenges. In the present study we have optimized a model of induced ischemic tolerance in the gerbil by carefully monitoring the duration of ischemic depolarization during each insult. Using this model we demonstrate that the threshold depolarization required to induce tolerance is comparable to those for induction of several transcription factor mRNAs, while mRNA encoding the heat shock protein, hsp72, is strongly induced only after more severe insults that approach the threshold for ischemic neuronal injury.

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INTRODUCTION

In recent years several investigators have described a phenomenon of induced ischemic tolerance, whereby brief ischemic or hyperthermic insults produce tolerance against later periods of more severe ischemia (Kitagawa et al. 1990, Kirino et al. 1991, Liu et al. 1992, Nishi et al. 1993, Simon et al. 1993). The initial studies were interpreted to suggest a role for proteins such as the 70 kDa heat shock protein, hsp72, in the tolerance phenomenon, based on the suggested protective role of the stress response following hyperthermic challenges (Pelham 1984, Johnston and Kucey 1988, Riabowol et al. 1988, Angelidis et al. 1991, Li et al. 1991), and the demonstrated induction of hsp72 in brain after even brief ischemic insults (Kirino et al. 1991, Simon et al. 1991).

The protection observed in most studies has been variable, and it has not been possible to predict with confidence the degree of tolerance to be expected in a given animal. Furthermore, it is well established that a wide range of changes in gene expression occur after ischemia, including the induction of proto-oncogene transcription factors that can regulate the expression of still other target genes (Nowak et al. 1990b, Kindy et al. 1991, Ikeda et al. 1994). There is some evidence that spreading depression, which typically does not induce hsp72 expression (Ikeda et al. 1994), may result in ischemic tolerance in some models (Kawahara et al. 1993, Kobayashi et al. 1995). We have recently shown that the duration of ischemic depolarization is a good predictor of the magnitude of inducible mRNA induction after brief ischemia in the rat (Halaby et al. 1993) (and manuscript in preparation). In the present study we have used this approach to compare depolarization thresholds for ischemic tolerance and gene expression in the gerbil.

METHODS

Mongolian gerbils (female, 50-70 g) were obtained from Tumblebrook Farm, West Brookefield, MA. Each animal was anaesthetized with 2% halothane in 70% N_2, 30% O_2 , the carotid arteries were exposed and looped with suture and the gerbil was fixed in a stereotaxic frame. Rectal temperature was maintained with a heating lamp under feedback control. Glass microelectrodes filled with 2 M potassium acetate were placed in each hippocampus to record DC potentials. The first ischemia was produced by applying tension to the suture loop for varied intervals of 0-5 min. The electrodes were then removed, the scalp incision was closed, and anaesthesia was maintained for 1 h.

For analysis of gene expression the animals were killed 1 h after the ischemic insult, brains were dissected, and frozen sections (16 μm) were collected for in situ hybridization. Sections were processed essentially as described previously (Nowak 1991), using oligonucleotide probes for hsp72 (Nowak et al. 1990a, Miller et al. 1991) as well as the proto-oncogenes c-*fos*, c-*jun*, *jun*B and *jun*D (Wisden et al. 1990), end labelled with α-[^{35}S]thio-dATP (Roychoudhury and Wu 1980). Images were obtained by autoradiography on Kodak SB5 film and quantitated by densitometry using calibrated ^{14}C standards (Amersham Life Sciences, Arlington Heights, IL).

For ischemic tolerance studies the animals were reanaesthetized 48 h after the first insult, electrodes were again placed in hippocampus, and a second test period of ischemia was produced that resulted in a standard depolarization interval of 6.5-8.5 min. Preliminary studies showed that this insult resulted in complete loss of CA1 neurones in naive animals. Gerbils were perfusion fixed with 10% formalin, 10% acetic acid, 80% methanol one week after the second insult. Paraffin sections were stained with hematoxylin-eosin and the density of CA1 neurones in dorsal hippocampus was determined by manual counting under light microscopy.

RESULTS

The potential variability in the ischemic impact of a given duration of carotid artery occlusion is illustrated by an extreme case in Fig. 1. There is a typical depolarization in the left hippocampus beginning approximately 1 min after the start of occlu-

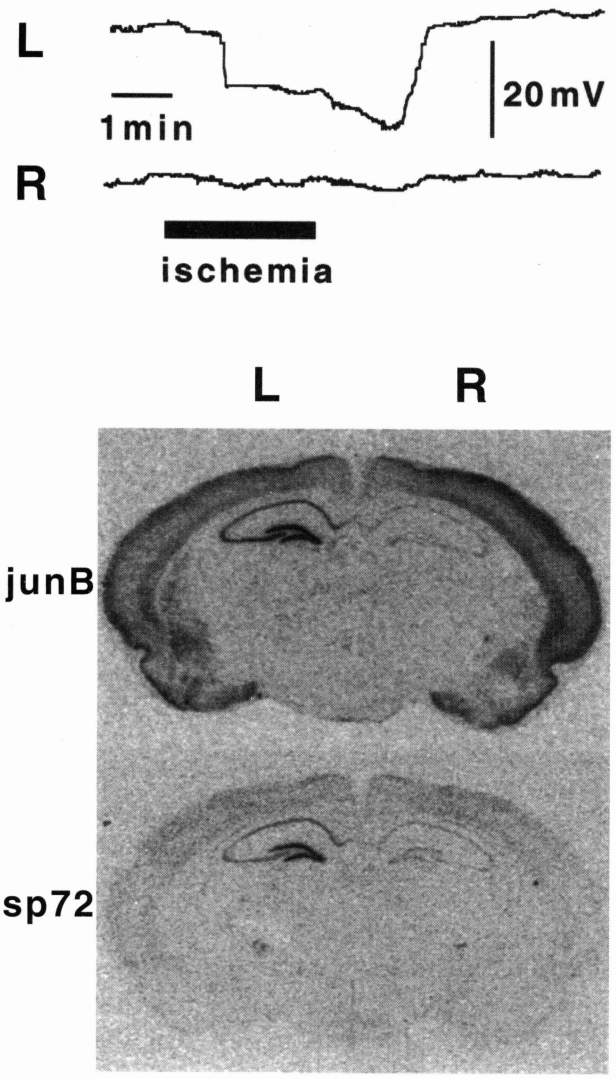

Fig. 1. Variability in hippocampal depolarization and gene expression following brief ischemia. Electrodes were implanted in each hippocampus of a gerbil which was then subjected to 2.5 min bilateral carotid artery occlusion and 1 h recirculation, followed by in situ hybridization analysis as described in the text. The upper panel demonstrates the onset and recovery of depolarization in the left hippocampus (L), while the right hippocampus (R) failed to depolarize. The lower panels illustrate changes in gene expression in the same animal, with induction of both *jun*B and hsp72 mRNAs restricted to the left, depolarized hippocampus. The hybridization results indicate that there was bilateral depolarization in cortex.

sion and recovering with a somewhat longer delay after recirculation. In contrast, the right hippocampus failed to depolarize in this animal. As illustrated for *jun*B and hsp72, the induction of ischemia-responsive mRNAs was absolutely dependent on ischemic depolarization. Interestingly, the right cortex showed significant expression of both mRNAs that is particularly obvious in the case of *jun*B, indicating that depolarization had occurred in cortex in the absence of an effective ischemic insult in hippocampus.

The duration of ischemic depolarization in a given hippocampus could be used quantitatively to determine the threshold insult required to produce a given effect. As indicated in Table I, depolarizations of 1.5-2.0 min during a priming occlusion resulted in a substantial but variable increase in CA1 neuron survival following a standard test insult administered 48 h later. Depolarizations of 2.0-2.5 min resulted in more pronounced protection, and animals that experienced 2.5-3.0 min depolarization had CA1 neurone counts that could not be distinguished from control animals. Longer insults resulted in a decline in cell number, presumably due to injury produced during the priming insult.

When depolarization thresholds for changes in gene expression were evaluated after 1 h recirculation in a separate group of animals it was evident that there was no detectable hsp72 expression in hippocampi that would have exhibited near maximal tolerance after 2.0-2.5 min depolarization (Table I). Studies at 6 h recirculation likewise indicated that less than 50% of hippocampi showed detectable hsp72 mRNA expression after depolarizations in the range that induced maximal tolerance (data not shown). Hsp72 mRNA levels in CA1 increased with increasing insult duration, reaching an apparent plateau after depolarizations of 5 min or longer. In contrast, the several transcription factor mRNAs all demonstrated half-maximal induction in the 2.0-2.5 min depolarization range that induced tolerance, with peak values observed after 3.0-5.0 min depolarizations, followed by a decrease after longer insults.

DISCUSSION

These observations demonstrate the possibility of obtaining predictable ischemic tolerance in the

TABLE I

Gene expression vs. depolarization duration after brief ischemia. Gerbils were subjected to transient ischemia with monitoring of ischemic depolarization in each hippocampus. One group of animals was subjected to a second test insult at 48 h and evaluated histologically after 1 week, and cell counts in CA1 for a given depolarization interval were determined as a percent of the nonischemic control value. In another group the levels of several induced mRNAs were evaluated at 1 h recirculation by in situ hybridization as described in the text, and expressed as a percent of the value for the depolarization interval demonstrating the maximum mean level. Values given are means ± standard deviation and the number of hippocampi (n) are indicated for each group

Depolarization interval (min)	0	0-1.5	1.5-2.0	2.0-2.5	2.5-3.0	3.0-4.0	4.0-5.0	>5.0
Tolerance study								
CA1 neurons (% control)	7±2	9±11	40±25	83±3	95±2	82±26	36±38	
	(8)	(4)	(14)	(10)	(7)	(12)	(5)	
Gene expression study (% maximum level)								
hsp72	1±3	1±2	0±3	1±5	13±5	20±20	50±30	100±12
c-*fos*	4±6	23±6	31±12	50±21	61±6	87±26	100±13	64±14
c-*jun*	9±6	20±10	24±12	55±30	92±25	100±31	88±29	65±13
*jun*B	0±3	9±74	48±21	62±26	61±18	100±12	80±11	44±17
*jun*D	7±7	21±9	24±17	45±21	64±7	86±24	100±25	88±16
	(10)	(5)	(5)	(7)	(5)	(5)	(7)	(5)

gerbil model. In addition, they clearly indicate the dissociation of hsp72 induction from ischemic tolerance, while suggesting the possibility that other responses related to the induction of proto-oncogene transcription factors could contribute to the tolerance effect.

The above results establish the importance of obtaining an independent measure of the duration of an ischemic insult following brief periods of occlusion. Previous studies have confirmed that ischemic intervals sufficient to result in energy failure and depolarization are required to induce both hsp72 and c-*fos*, but did not specifically examine the duration of depolarization following brief insults (Ikeda et al. 1994). Other studies established that even brief depolarizations such as those produced during spreading depression were sufficient to induce c-*fos* and other immediate-early genes, while hsp72 expression was not induced under such conditions (Welsh et al. 1992, Ikeda et al. 1994, Kinouchi et al. 1994, Nowak and Jacewicz 1994). The present results quantitatively demonstrate the dissociation of the depolarization threshold for the stress response from that of many other postischemic changes in gene expression in gerbil brain, and a similar dissociation is evident in the rat (Halaby et al. 1993).

Signalling mechanisms triggered by depolarization and transient calcium influx have been identified to account for increases in immediate-early gene expression following various stimuli (Sheng and Greenberg 1990, Sheng et al. 1990). While there is evidence that calcium influx may also contribute to induction of the heat shock response, the cascades responsible for stress protein induction remain to be fully established (Price and Calderwood 1991). In the context of ischemia it is clear from the present results that events occurring with some delay after depolarization must be responsible for transducing the heat shock response. As a matter of practical importance, these studies confirm that hsp72 can be induced to some extent after ischemic insults that result in minimal neuronal injury (Kirino et al. 1991, Simon et al., 1991), but the data of Table I clearly indicate that more pronounced induction of stress protein mRNA occurs after insult durations associated with CA1 neuron loss. Recent pharmacological studies suggest that ATP-sensitive K^+

channels and adenosine release contribute to both postischemic changes in gene expression and ischemic tolerance (Heurteaux et al. 1995), and it would be of interest to investigate these effects in the present model.

These results demonstrate that widespread changes in gene expression are associated with the production of insults that induce tolerance, and the challenge remains to determine which, if any, of these reponses may be involved in the tolerance mechanism. There is evidence that induced mRNAs of the *jun* family are successfully translated after brief insults that can induce tolerance (Nowak et al. 1993, Sommer et al. 1995), but the functional consequences of this expression have yet to be established. Recent studies indicate that *bcl*-2, which has been shown to protect against cell death by various mechanisms, may also be expressed in tolerant neurons (Shimazaki et al. 1994). A wide range of ischemia-inducible mRNAs such as superoxide dismutase (Matsuyama et al. 1993), and growth factors such as BDNF (Lindvall et al. 1992) could also be induced and translated under conditions of ischemic tolerance. There is evidence that heat shock mRNAs are more transiently induced in tolerant animals, and improved protein synthesis recovery may allow hsp72 mRNA to be more readily translated at the time of the test insult (Aoki et al. 1993, Furuta et al. 1993, Nakagomi et al. 1993, Kanemitsu et al. 1994). While the present results have established that prior hsp72 induction is not required for tolerance, this leaves open the possibility that the stress response may yet be found to play a role in tolerance *via* more effective heat shock protein induction in response to the acute ischemic test challenge. The availability of a predictable model should now allow further experimental evaluations of the mechanisms of induced tolerance following brief ischemia.

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