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Research paper

Sensorineural hearing loss and ischemic injury: Development of animal models to assess vascular and oxidative effects

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ABSTRACT

Hearing loss may be genetic, associated with aging or exposure to noise or ototoxic substances. Its aetiology can be attributed to vascular injury, trauma, tumours, infections or autoimmune response. All these factors could be related to alterations in cochlear microcirculation resulting in hypoxia, which in turn may damage cochlear hair cells and neurons, leading to deafness. Hypoxia could underlie the aetiology of deafness, but very few data about it are presently available. The aim of this work is to develop animal models of hypoxia and ischemia suitable for study of cochlear vascular damage, characterizing them by electrophysiology and gene/protein expression analyses. The effects of hypoxia in infarction were mimicked in rat by partial permanent occlusion of the left coronary artery, and those of ischemia in thrombosis by complete temporary carotid occlusion. In our models both hypoxia and ischemia caused a small but significant hearing loss, localized at the cochlear apex. A slight induction of the coagulation cascade and of oxidative stress pathways was detected as cell survival mechanism, and cell damages were found on the cuticular plate of outer hair cells only after carotid ischemia. Based on these data, the two developed models appear suitable for *in vivo* studies of cochlear vascular damage.

1. Introduction

Hypoxia plays a relevant role in heart, cerebrovascular, chronic lung and peripheral vascular diseases and also in cancer and neuron death. Its aetiology is often related to vascular and ischemic damage in which O_2 and energy substrate delivery together with metabolite removal are involved. The basic effects of hypoxia are exerted by depriving cells of adequate amounts of O_2 to meet their metabolic needs. Hypoxia induces the expression of several genes involved in the adaptation to decreasing O_2 (Jeong et al., 2005), such as those of erythropoietin (Jelkmann, 1992), vascular endothelial growth factor (Goldberg and Schneider, 1994) and glycolytic enzymes (Semenza et al., 1994).

Experimental and clinical studies have shown that ischemia could be a consequence of noise-induced hearing loss, since cochlear oxygen tension is reduced during and after noise exposure (Thorne and Nuttall, 1987; Scheibe et al., 1993, 1997; Lamm and Arnold, 1996; Chen, 2002; Orita et al., 2002). Alterations in blood flow have also been detected in sudden sensorineural hearing loss induced by noise and presbyacusis, therefore ischemia maybe involved. Anoxia or reperfusion leads to cochlear dysfunction by inflammatory factors, among which nitric oxide that can affect cochlear outer hair cells (OHC) (Tabuchi et al., 1999; Jung et al., 2003; Jeong et al., 2005a,b).

Although an extensive literature is available on cochlear ischemia (Lin et al., 2010) and neonatal hypoxia leading to hearing loss (Mwaniki et al., 2012), few studies deal with the relationship of hearing loss to hypoxia in adults and carotid ischemia. The carotid system is not directly related to cochlear blood supply, nevertheless Shirane and Harrison (1987) found stereocilia disorganization and cytoplasmic protrusions in hair cells of chinchillas subjected to hypoxia. With increasing hypoxia severity, the damages occurred

Abbreviations: ABR, auditory brain response; Bcl-2, B-cell lymphoma-2; cyt-c, cytochrome *c*; HIF-1α, hypoxia-inducible factor 1α; IHC, inner hair cells; JNK, c-Jun N-terminal kinase; pJNK, phospho c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MYH-6, myosin 6; NF-200, neuro-filament 200; NF-kB, nuclear factor-kappa beta; OHC, outer hair cells; ROS, reactive oxygen species; TSH, threshold shift; TF, tissue factor; TM, thrombomodulin

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first in the inner hair cells (IHC) and then in OHC. However, Cheng et al. (1999) noticed in a rat *in vitro* model of organ of Corti a decrease in hair cell number after ten hours of hypoxia, but both IHC and OHC were equally sensitive to hypoxia, with damage increasing from the apex to the base.

The factors causing hair cells loss in ischemia are poorly understood. Mazurek et al. (2003) showed that the combination of glucose deprivation and oxygen deficiency could dramatically increase the loss of OHC and IHC. In apolipoprotein E knock-out mice it was found that atherosclerotic plaques were associated with deafness, which improved after pharmacological treatment (Cai et al., 2009). These data were confirmed by clinical studies on humans, showing that an inadequate blood supply in the inner ear due to atherosclerosis could cause hearing loss (Yoshioka et al., 2010).

All mid- or long-term cell responses require changes in gene expression: recent research suggests that most human cells are able to perceive and respond to hypoxia (Wang and Semenza, 1993; Semenza, 1996, 2001).

Hypoxia-inducible factor 1α (HIF-1α) is a normally degraded transcription factor which becomes stable only during hypoxia, forming a dimer with hypoxia-inducible factor 1β which activates oxygen-sensitive proteins (Semenza et al., 1994; Yamashita et al., 2001; Liu et al., 2002). The expression of HIF-1α correlates with the activation of mitogen-activated protein kinases (MAPK) by hypoxia in many cells (Shemirani and Crowe, 2002; Mottet et al., 2003; Jeong et al., 2005). MAPK are major components of pathways controlling embryogenesis, cell differentiation, cell proliferation, and cell death (Pearson et al., 2001). The c-jun N-terminal kinase (JNK) is activated by mitogens and also by a variety of environmental stresses (heat shock, ionizing radiation, oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), ischemia-reperfusion injury, mechanical shear stress, vasoactive peptides, proinflammatory cytokines, pathogen- and damage-associated molecular pattern protein (PAMPs/DAMPs) and translational inhibitors (cycloheximide and anisomycin) (Dérijard et al., 1994; Kyriakis et al., 1994; Weston and Davis, 2007; Kyriakis and Avruch, 2012).

The aim of this study is to develop rat animal models suitable for the study of hypoxic and ischemic damage of the inner ear, and characterize them by electrophysiology and gene/protein expression analyses. For this purpose we applied in the rat model the hypoxia effects by partial permanent obstruction of the left coronary artery (as in infarction), and the ischemia effects by complete temporary carotid occlusion (as in thrombosis). Since in literature there are no detailed studies about the relationship between coagulation factors and hypoxia or ischemia in the inner ear, we analysed factors involved in coagulation, oxidative stress and apoptosis in rat organ of Corti, stria vascularis and spiral ganglion.

2. Material and methods

2.1. Animals

Forty-two male Sprague Dawley rats (150–200 g; Charles River, Italy) were used in this study. The animals were treated according to Italian guidelines DL 116/92 with reference to European Economic Community directive 86-609, and the study was approved by the local animal care ethic committee. The animals were randomly assigned to 7 groups treated as follows: untreated control animals (A, N: 5); temporary carotid artery occlusion (ischemia) for 5 min (B, N: 10), for 10 min (C, N: 10) and for 15 min (D, N: 2); partial permanent obstruction of the left coronary artery (hypoxia) and sacrifice after 3 h (E, N: 5), after 1 week (F, N: 5) and after 1 month (G, N: 5).

2.2. Measurement of evoked potentials

Auditory brainstem responses (ABR) were used to assess the auditory threshold. All ABR tests were performed as described by Cascella and coworkers (2012), before surgery and before the sacrifice. Briefly, animals were pre-treated with an anaesthetic solution composed by 50 mg tiletamine (Zoletil 100, Virbac, Milan, Italy) in 1-mL physiological saline to which 0.5 ml 2% xylazine (Rompun, Bayer Milan, Italy) was added.

2.3. Surgical procedures

Animals were anesthetized and treated as follows. Common carotid arteries were exposed bilaterally and totally occluded for 5, 10 or 15 min by clamps. The clamp was then removed and the incision site was sutured with 5-0 silk thread. After 3 h of reperfusion the animal was sacrificed.

The coronary ischemic stroke was caused by partial permanent occlusion of left coronary artery was performed as modified from Chen et al. (2001). The animals were anesthetized, tracheotomized and connected to an apparatus to promote artificial respiration. A thoracotomy was performed on the left side in the intercostal space between the fourth and the fifth rib, and the heart was exposed. Myocardial ischemia was then induced by partial occlusion of the left coronary artery, sutured with a 6-0 silk thread. The incision site was then sutured with 5-0 silk thread. The operated animals were anaesthetized and painlessly sacrificed by decapitation respectively 3 h, 1 week or 1 month after surgery. After temporal bone removal, the bulla was opened to expose the otic capsule, and cochleae were explanted and collected for quantitative PCR and histological analyses.

2.4. Quantitative Real-Time PCR

One cochlea from each animal (for a total of 40) was homogenized and preserved in RNAlater ® (Applied Biosystems, Life Technologies, Monza, Italy) and RNA extraction was performed with Trizol ® (Life Technologies, Monza, Italy). The RNA samples were then incubated with DNase I (New England Biolabs, Hitchin, UK) to remove DNA contamination, and was then reverse transcribed to cDNA with iScript ™ Reverse Transcription Supermix (Bio-Rad, Milan, Italy). After measuring RNA concentration by a Nanophotometer ® P300 (Implen, Munich, Germany), a quantitative Real Time PCR for genes of interest (Tissue factor, TF; Thrombomodulin, TM; Hypoxia-inducible factor 1 α, HF-1α; c-Jun N-terminal kinase, JNK; B-cell lymphoma-2, Bcl-2; Nuclear factor kappa-B, NF-kB; caspase-3) was performed in the thermocycler Chromo-4 System (Bio-Rad) with Sso-fast ™ EvaGreen ® Supermix (Bio-Rad). We used β-actin, Tata-binding protein and beta-2-microglobulin as reference genes for normalization performed via the software GenEx (©2004, Bio-Rad), which uses algorithms from Vandesompele et al. (2002) and geNorm software (© 2007–2012 Biogazelle Zwijnaarde, Belgium). The cycling parameters were: 98 °C for 30 s, 39 cycles of 98 °C for 2 s and 60 °C for 7 s. Data were plotted as fold increase compared to the control (set: 1). Each experiment was performed in triplicate.

2.5. Histology

One cochlea from each animal (for a total of 42) was processed for paraffin inclusion. Under a stereomicroscope SMZ1000 (Nikon Instruments, Florence, Italy), the cochlear apex was drilled to promote fixation with Shandon Glyo-Fixx™ (Thermo Scientific, Milan, Italy). The cochleae were soaked overnight in fixative at 4 °C and then washed in 0.1 M PBS, pH 7.4 (Lonza, Milan, Italy). The