Research Report

Expression of apoptosis-related genes in the organ of Corti, modiolus and stria vascularis of newborn rats

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ABSTRACT

Cell death in the inner ear tissues is an important mechanism leading to hearing impairment. Here, using microarrays and real-time RT-PCR we analyzed expression of selected apoptosis-related genes in rat’s inner ear. We determined the gene expression in tissues freshly isolated from neonatal rats (3–5 days old) and compared it to that of explants cultured for 24 h under normoxic or hypoxic conditions. For the analyses, we used pooled samples of the organ of Corti (OC), modiolus (MOD) and stria vascularis (SV), respectively. We observed region-specific changes in gene expression between the fresh tissues and the normoxic culture. In the OC, expression of the proapoptotic genes caspase-2, caspase-3, caspase-6 and calpain-1 was downregulated. In the MOD, the antioxidative defense SOD-2 and SOD-3 were upregulated. In the SV, caspase-2, caspase-6, calpain-1 and SOD-3 were downregulated and SOD-2 upregulated. We speculate that these changes could reflect survival shift in transcriptome of inner ear explants tissues under in vitro conditions. With the exception of SOD-2, hypoxic culture conditions induced the same changes in gene expression as the normoxic conditions indicating that culture preparation is likely the dominating factor, which modifies the gene expression pattern. We conclude that various culture conditions induce different expression pattern of apoptosis-related genes in the organotypic cochlear cultures, as compared to fresh tissues. This transcriptional pattern may reflect the survival ability of specific tissues and could become a tempting target for a pharmacological intervention in inner ear diseases.

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Abbreviations: BSG, buffered saline glucose; HIF, hypoxia inducible factor; IHC, inner hair cell; LDH, lactate dehydrogenase; MOD, modiolus; NF 200, neurofilament 200 kDa; OC, Organ of Corti; OHC, outer hair cell; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SGN, spiral ganglion neurons; SOD, superoxide dismutase; SV, stria vascularis

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1. Introduction

Inner ear is a very complex organ containing within cochlea the organ of Corti (OC) with its neurosensory hair cells, the modiolus (MOD) including the spiral ganglion neurons and the stria vascularis (SV) specialized to maintain the inner ear homeostasis. The cochlea registers and amplifies the external mechanical signals and signals to the central nervous system via the spiral ganglion neurons. Damage of the inner ear structures results in two functional impairments: hearing loss and/or tinnitus. Both are among the most frequent disorders of the neurosensory system with a prevalence of about 10% of the population (Gates and Mills, 2005).

In cochlea, OC, MOD and SV act cooperatively and can be injured independent of each other. For example, hypoxia/ischemia affects not only hair cells but also afferents and the SV. Perinatal and postnatal hypoxia and asphyxia are associated with severe atrophy of the organ of Corti and with a necrosis of the hair cells in the OC or the neurons in MOD, which belong to the most vulnerable cells in the cochlea (Cheng et al., 1999; Mazurek et al., 2003). Once the hair cells or neurons are damaged or lost, they cannot be replaced.

Studies have shown that members of several protein families like superoxide dismutases (SODs), Bcl-2, caspases (Casp) and calpains (Calp) contribute substantially to the cell death in the inner ear (Cheng et al., 1999, 2005; Feghali et al., 1998; Henderson et al., 2006; Lee et al., 2004; Lefebvre et al., 2002). Reactive oxygen species (ROS) play an important role in hair cell survival and in the induction of apoptosis (Lefebvre et al., 2002). The most important line of antioxidative defence systems against ROS and particularly the superoxide anion radicals are the enzymes of the SOD family. Three distinct isoforms of SOD have been identified in the tissues of aerobic organisms (Zelko et al., 2002). Two isoforms have Cu and Zn in the catalytic center and are localized in the cytoplasm (SOD-1) or in the extracellular fluid (SOD-3). SOD-2 has manganese in the catalytic center and is localized in the mitochondria. Cu–Zn–SOD and Mn–SOD protein are heterogeneously distributed in the SV, spiral ligament and OC. High concentrations of Cu/Zn–SOD and Mn–SOD were found in the SV but low concentrations in the OC (Yao and Rarey, 1996). SOD-3 was first detected in extracellular fluids like lymph, ascites and cerebrospinal fluid. Its expression is highly restricted to specific tissues and cells (Nozik-Grayck et al., 2005).

The Bcl-2 protein family regulates the cell intrinsic apoptotic pathway. The members of the Bcl2-family comprise anti- and proapoptotic proteins (Kim, 2005; Lindsten et al., 2005). The major anti-apoptotic family proteins are Bcl-2 and Bcl-xL (=Bcl2-like 1). Proapoptotic Bcl-2 gene products participate in triggering apoptosis by interacting with mitochondrial membrane and the release of proteins from the mitochondrial intermembrane space, including cytochrome c (Soane and Fiskum, 2005). The two major proapoptotic family members are Bax and Bak (Lindsten et al., 2005). Several splice variants of Bax have been found (Jin et al., 2001).

Caspases and calpains are important factors for the initiation and execution of cell death in the inner ear (Lefebvre et al., 2002; Nicotera et al., 2003). Caspases are aspartate-specific cysteine proteases, which normally exist as inactive precursor molecules. Caspase-mediated proteolysis plays a critical role in apoptosis, either as initiator caspases (e.g., caspases-1, -2) or as downstream effector caspases (e.g., caspases-3, -6) (Fan et al., 2005; Fuentes-Prior and Salvesen, 2004; Van de Water et al., 2004). Marked differences in the expression profiles of the various caspase mRNA following ischemia of the brain were described (Harrison et al., 2001).

Calpains are calcium-activated cysteine proteases, which in an intact cell exist in an inactive form. At least 14 mammalian calpains have been identified, among them two ubiquitous calpains (calpain-1 and calpain-2). Calpain-1 (=μ-calpain) and calpain-2 (=m-calpain) are distinguished by the optimal calcium concentration for maximal activity (Huang and Wang, 2001; Wang, 2000). Calpain-1 shows half-maximal proteolytic activity at Ca++ 0.5–2 μM, whereas calpain-2 at 400–800 μM. Calpains are involved in apoptosis because of their ability to cleave and thus activate several proteins that regulate progression of apoptosis, including caspases. Inhibitors of calpains were found to protect the auditory sensory cells from hypoxia-induced cell and tissue damage (Cheng et al., 1999).

Cultures of the different regions of the inner ear are valuable models to study the vulnerability of specific cells to various ototoxic conditions, including hypoxia/ischemia. The classic model to study the hair cell loss in the OC is the organotypic culture (Lowenheim et al., 1999; Sobkowicz et al., 1993). To study the loss of spiral ganglion neurons (SGN) or of SV cells, explant and dissociated primary cell cultures are commonly used (Lefebvre et al., 1991; Whitlon et al., 2006; Kim et al., 1996; Mou et al., 1997a). In these cultures, long-term (days and weeks) survival of the neurosensory hair cells and of the neurons of the spiral ganglion and of SV depends on the addition of region-specific growth factors.

The aim of our study was to analyze the expression of apoptosis-related, selected genes in the inner ear of newborn rats. We hypothesized that changes in transcriptome occurring in normoxic culture and in culture subjected to a mild hypoxic episode could reveal region-specific intrinsic cellular mechanisms, which enable inner ear tissues to survive stress situations (i.e., mechanical dissection of the cochlea or hypoxia). We determined the mRNA expression in OC, MOD and SV using expression microarray chip for primary screening and a real-time RT-PCR for in-detail confirmation. To specifically analyze expression of genes associated with the neurosensory system, we used the neurobiological chip RN-U34, which targets selected 1322 gene transcripts.

2. Results

To ensure the integrity of tissue and cells in culture, we used biochemical and morphological criteria. First, we measured the release of lactate dehydrogenase (LDH), which is normally released from cells with damaged membranes. We observed
no differences in the activity levels of LDH indicating a non-significant level of cell death (Supplementary Fig. 1). Similarly, the expression of neurofilament 200 (NF 200) mRNA was not altered, indicating neuronal structures (Supplementary Fig. 2). The live/dead cytotoxicity test using propidium iodide (PI) and calcein AM showed bright green staining with some PI-stained nuclei indicating a viable status of majority of the cells (Supplementary Fig. 3). The live/dead test using Hoechst 33342 and PI analyzed with a use of confocal microscopy confirmed the viability of normoxic cultures after 24 h (Supplementary Fig. 4). Using the same methods, we showed that hypoxia induces a moderate level of cell damage in the organotypic cultures. To characterize the viability of hair cells, we determined the number of intact outer and inner hair cells in the OC and presented it in the cochleogram (Fig. 1).

In the controls and the normoxic cultures, we counted 9.9±2.0 (mean ± SD) IHCs/100 μm and 13.6±5.0 OHCs/100 μm in each row, indicating that the normoxic culture itself has no damaging effect on hair cells. Hypoxic conditions, however, induced a hair cell loss within a range of 30–50% of IHCs, mainly in the basal part of the cochlea, and 20% of OHCs.

Fig. 1 – Numbers of inner and outer hair cells (IHCs/OHCs) counted in the organ of Corti of newborn rats. The percent distance from the apex is plotted on the x-axis; y-axis indicates the numbers of IHCs (top) or OHCs (bottom) per 100-μm sections of the organ of Corti. Co: control (freshly isolated and immediately processed tissue); Cu-No: 24 h organotypic cultures under normoxic conditions; Cu-Hy: 24 h organotypic cultures that included 5 h of hypoxia and the rest normoxic conditions. Plotted are means ± SEM (n=9). There were no statistically significant differences between controls and normoxic cultures (Scheffé’s test). IHC: Co versus Cu-Hy, p<0.001; OHC: Co versus Cu-Hy, p<0.006.

Taken together, above findings demonstrate that organotypic cultures of OC, Mod and SV maintain the viability under normoxic conditions. In contrast, hypoxia exposure induces moderate cell damage.

Microarray expression analyses showed a statistically significant expression of 16 apoptosis-related genes in the control and/or in the experimental samples. For 11 selected genes, we performed additional analyses using real-time RT-PCR. Table 1 contains the numerical data from the microarray transcriptome analyses of the control samples, expressed as normalized signals (NS). Additionally, in parenthesis presented are the real-time RT-PCR data, expressed as arbitrary units (AU). Cluster analysis (k-mean; data not shown) of the data obtained from microarrays and from RT-PCR showed that SOD-1 and calpain-1 are the genes with the highest expression in all regions. Further analyses revealed a statistically significant positive correlation between the data generated by microarray and by real-time RT-PCR using the standard curve method and the comparative CP method (Fig. 2). Table 2 compares the fold changes, obtained from the microarray and the RT-PCR analyses. We found a statistically significant correlation between the microarray and the real-time RT-PCR fold change data. All our further analyses presented in this paper are based on real-time quantitative RT-PCR data, a standard method for the analysis of mRNA levels (Figs. 3–6). To better illustrate the differential gene expression, control OC tissue sample was used as a reference.

Fig. 3 compares the relative expression of cytosolic SOD-1, mitochondrial SOD-2 and extracellular SOD-3 in the control tissues (OC, Mod and SV) and its respective modulation in normoxic and hypoxic cultures. The cytosolic SOD-1 was not changed in a statistically significant manner. There was a similar basal expression of the mitochondrial SOD-2 in OC, Mod and SV, which increase in OC after hypoxia and in Mod after 24 h of normoxic and hypoxic culture conditions. An increase in the expression of SOD-2 was also found in the SV, however, the hypoxia-induced transcript level was lower as compared to the normoxic cultures. The transcription of extracellular SOD-3 was higher in SV as compared to OC and Mod in the control samples. Both, hypoxic and normoxic culture conditions increased the SOD-3 transcript expression level in Mod, while decreasing it in SV.

The microarray chip includes three Bax and two Bcl-2I gene homologues with an expression level classified as being present (Table 1). Two Bax homologues and one Bcl2I gene were selected for the RT-PCR confirmation. Microarray and RT-PCR data indicated that expression of Bax homologue U49729 was upregulated in MOD during culture, whereas in SV and OC its expression remained unchanged. In contrast, expression of Bax homologue U59184 was not changed. Expression of Bcl2I gene was downregulated during normoxic and hypoxic culture in MOD and SV. Interestingly, Bcl2I was expressed on the highest level in the freshly isolated control samples of SV, as compared to those of OC and MOD (Fig. 4).

Three gene homologues of caspase (caspase-1, -2, -3, -6, -7) and five homologues of calpain gene family (calpain-1, -2, -3, -8, -9) are included on the microarray chip. Caspase-2, -3, -6 and calpain-1 were selected for the RT-PCR confirmation. The following regional and conditional features in the expression of these genes were observed (Fig. 5): (1) Caspase-2 expression...
decreased in normoxic and hypoxic cultures in all regions studied. (2) Caspase-3 expression decreased in OC and SV in normoxic and hypoxic cultures in all regions studied. (3) Caspase-6 expression decreased in OC and SV in hypoxic cultures and increased in MOD cultures. (4) Calpain-1 expression decreased in OC and SV in normoxic and hypoxic cultures.

Relevant evidence suggests that the cellular prion protein (Prnp) is involved in ischemic brain injury and apoptosis (Roucou et al., 2005; Spudich et al., 2005). Microarray data showed an unexpectedly high expression, specifically in MOD (Table 1). Because the Prnp expression in the inner ear is described here for the first time, we reanalyzed its expression by real-time RT-PCR (Fig. 2). As expected based on the microarray data, MOD showed the highest, OC medium and SV the lowest expression level. There were no changes in Prnp gene expression in hypoxic or normoxic cultures.

The complete data sets from this study have been deposited according to the MIAMI standard to Gene Expression Omnibus (GEO) band and can be accessed by ID GSE5446.

### 3. Discussion

In this paper, we have analyzed expression of selected, apoptosis-related genes in the inner ear tissues that were either freshly isolated or cultured for 24 h under normoxic or hypoxic conditions. Using two independent techniques, we found that organotypic culture under normoxic conditions is associated with a region-specific expression modulation of pro- and anti-apoptotic genes, possibly indicating an effective adaptation to the in vitro conditions. The successful adaptation to culture is best illustrated by the survival of all highly vulnerable hair cells (Fig. 1).

#### Table 1 – Expression of genes analyzed by microarray and real-time RT-PCR in freshly prepared tissue samples from 3- to 5-day-old rats (controls)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Organ of Corti</th>
<th>Modiolus</th>
<th>Stria vascularis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sod-1 (M21060)</td>
<td>36400 (5895 ± 864)</td>
<td>27076 (4059 ± 707)</td>
<td>33912 (4697 ± 773)</td>
</tr>
<tr>
<td>Sod-2 (Y00497)</td>
<td>1578 (1290 ± 197)</td>
<td>1528 (1118 ± 276)</td>
<td>1782 (1494 ± 191)</td>
</tr>
<tr>
<td>Sod-3 (Z24721)</td>
<td>11203 (677 ± 67)</td>
<td>6659 (572 ± 134)</td>
<td>32227 (1267 ± 231)</td>
</tr>
<tr>
<td>Bax (57611)</td>
<td>2380</td>
<td>1392</td>
<td>3851</td>
</tr>
<tr>
<td>Bax (U49729)</td>
<td>651 (759 ± 116)</td>
<td>900 (674 ± 165)</td>
<td>790 (772 ± 100)</td>
</tr>
<tr>
<td>Bax (US5184)</td>
<td>28913 (108 ± 64)</td>
<td>36745 (95 ± 40)</td>
<td>26772 (110 ± 54)</td>
</tr>
<tr>
<td>Bcl21 (S72824)</td>
<td>1823</td>
<td>1560</td>
<td>2376</td>
</tr>
<tr>
<td>Bcl21 (U39663)</td>
<td>1370 (252 ± 58)</td>
<td>1360 (193 ± 58)</td>
<td>1690 (418 ± 145)</td>
</tr>
<tr>
<td>Casp1 (S79676)</td>
<td>1783</td>
<td>2714</td>
<td>2872</td>
</tr>
<tr>
<td>Casp2 (U77933)</td>
<td>7589 (210 ± 47)</td>
<td>14684 (269 ± 40)</td>
<td>12059 (189 ± 89)</td>
</tr>
<tr>
<td>Casp3 (U49930)</td>
<td>872 (247 ± 82)</td>
<td>300 (145 ± 16)</td>
<td>331 (192 ± 38)</td>
</tr>
<tr>
<td>Casp6 (AF025670)</td>
<td>5638 (156 ± 52)</td>
<td>3133 (66 ± 30)</td>
<td>6552 (185 ± 55)</td>
</tr>
<tr>
<td>Capn1 (U58359)</td>
<td>42338 (3045 ± 159)</td>
<td>43104 (3067 ± 674)</td>
<td>51201 (3089 ± 191)</td>
</tr>
<tr>
<td>Capn2 (L09120)</td>
<td>6281</td>
<td>6881</td>
<td>5486</td>
</tr>
<tr>
<td>p53 (X13058)</td>
<td>9877</td>
<td>12478</td>
<td>16367</td>
</tr>
<tr>
<td>Prnp (D50093)</td>
<td>11679 (842 ± 191)</td>
<td>24020 (1648 ± 248)</td>
<td>5071 (224 ± 57)</td>
</tr>
<tr>
<td>Numbers indicate the mean values of the normalized signals (microarray). The numbers in brackets indicate real-time RT-PCR data expressed in arbitrary units (AU, see Experimental procedure), mean ± SD, n=6. The microarray analysis classified the following genes as absent: caspase-7 (AF072124), calpain-3 (AF052540, AF061726, J05121), calpain-8 (D14478, D14480), and calpain-9 (U89514). Bcl-2-related ovarian killer protein (Af072954); Bcl-2-like11 (apoptosis facilitator, Bcl2l11, AF065431/2/3); Bcl-2-associated death promoter (Bad, AFO05523); B-cell leukemia/lymphoma (Bcl2, L14680, S74122); Bcl-2-like1 (AF096291); Death effector domain (Dedd, AF053362); Fas apoptotic inhibitory molecule 2 (AF044201); BHS interacting (with Bcl2 family) domain and apoptosis agonist (Bid3, D83697, E13573cds).</td>
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</table>

#### 3.1. Consistency of microarray with RT-PCR data

To estimate the variations between microarrays and RT-PCR, we performed real-time RT-PCR using aliquots of the same RNA samples taken for the microarray analyses. Correlation coefficients within the range of $R^2=0.82$–0.87 were observed (three different genes, each 12 samples, data not shown), which correspond to a coefficient of determination level of about 91–93%. In contrast, microarray and real-time RT-PCR data obtained from different biological experiments resulted in a correlation coefficient of $R^2 = 0.53$ to 0.71, corresponding to a coefficient of determination of 70–80%. Therefore, we arrived at the conclusion that the variation between the microarray and present RT-PCR data is of biological and not of methodological nature.

To further analyze the consistency between the microarray and the real-time RT-PCR data, we normalized the differential expression of the genes using a reference gene. Because hypoxia affects most of the so-called housekeeping genes, we used SOD-1 gene as reference (Zhong and Simons, 1999). As shown in Tables 1 and 2, SOD-1 is the only gene which did not show significant expression changes between the control and experimental samples, irrespective of being analyzed by microarray or by real-time RT-PCR. The standard curve method as well the comparative CP method resulted in a similar correlation coefficient indicating that both approaches could be used for quantification of gene expression (Fig. 2).

Of 16 apoptosis-related genes identified as significantly expressed in cochlear tissues with the use of microarray, expression level of 11 genes was assessed using real-time RT-PCR. Expression of one gene (Bax US5184) did not correlate with the microarray data, regardless of the evaluation procedure used (relative expression level, fold change). To explain this
Correlation between the data obtained from microarray and from real-time RT-PCR. Top panel: correlation between the normalized signals (NS, microarray) and the arbitrary PCR units (AU, real-time RT-PCR) using the standard curve method. This correlation is represented by the equation $y = 0.10x + 0.10$ ($n=90$, $R^2 = 0.71$, $p<0.01$). A similar correlation was calculated for the comparative CP method using the expression value of SOD-1 as a reference gene ($R^2 = 0.65$; $p<0.01$, $n=86$). The Bax (U59184) gene appeared to be an outlier and was not included in these analyses. Bottom panel: correlation between the fold changes analyzed by microarray and the fold changes analyzed by real-time RT-PCR. This correlation is represented by the equation $y = 0.19x + 0.53$ ($n=60$, $R^2 = 0.53$, $p<0.01$).

Fig. 2 – Correlation between the data obtained from microarray and from real-time RT-PCR. Top panel: correlation between the normalized signals (NS, microarray) and the arbitrary PCR units (AU, real-time RT-PCR) using the standard curve method. This correlation is represented by the equation $y = 0.10x + 0.10$ ($n=90$, $R^2 = 0.71$, $p<0.01$). A similar correlation was calculated for the comparative CP method using the expression value of SOD-1 as a reference gene ($R^2 = 0.65$; $p<0.01$, $n=86$). The Bax (U59184) gene appeared to be an outlier and was not included in these analyses. Bottom panel: correlation between the fold changes analyzed by microarray and the fold changes analyzed by real-time RT-PCR. This correlation is represented by the equation $y = 0.19x + 0.53$ ($n=60$, $R^2 = 0.53$, $p<0.01$).

discrepancy we performed BLAST sequence alignment of the target sequence used by Affymetrix and our sequenced amplicon (data not shown). The amplicon was 100% homologous to the reference gene (AF235993) (Jin et al., 2001), 100% homologous to the Affymetrix Bax target (U59184) and 97% homologous to Bax sequence with accession No. U49729. The reason for the dramatic differences in the expression level of the Bax (U59184) determined by microarray and RT-PCR is not clear (Table 1).

Altogether, the microarray analyses of two pooled samples proved to be a valuable and meaningful screening approach for transcriptome analysis. However, we recommend confirmation of microarray results using real-time RT-PCR as we did for our most interesting targets because the second method generates quantitative and more accurate results.

3.2. Culture-induced changes in gene expression

There are several ways to culture OC, MOD and SV: between others explant cultures and dissociated cultures. We have demonstrated that OC, MOD and SV isolated and cultured as explants (also designated “organotypic cultures”) maintain vital status for at least 24 h without region-specific media or growth factors. The advantage of an organotypic culture over the dissociated culture is preservation of the anatomical structure that allows comparing the endogenous tissue response to injury. We selected the 24-h time point to study the changes in gene expression because of our observation that under normoxic conditions nearly all cells are viable after 24 h whereas a clear cell loss occurs at that time after exposure to hypoxia. Nevertheless, because the time window for the activation of various genes may be different, caution should be taken in extrapolation of the results of this study to an in vivo situation (Kirkegaard et al., 2006). We also want to point out that the subdivision during preparation of cochlear tissues may affect the experimental outcome. The inner ear has an apical–basal gradient of sensitivity to injury. This gradient could mask subtle expression differences. However, based on our previous work, we expect approximately similar changes in gene expression in the apical and basal part (Gross et al., 2005).

3.2.1. Organ of Corti

The normoxic culture of OC is associated with an expressional downregulation of the proapoptotic genes caspase-2, caspase-3, caspase-6 and calpain-1. Because the proteins encoded by these genes play an important role in executing apoptotic cell death, expressional changes could reflect an effective way of adaptation to damaging conditions resulting in a decrease of apoptotic activity (Harrison et al., 2001). Caspase-2 is an initiator of apoptosis, sensing changes in mitochondrial potential (Chong et al., 2005; Fan et al., 2005; Fuentes-Prior and Salvesen, 2004). This pathway includes release of cytochrome c and subsequent activation of caspase-9 and caspase-3. Because of alternative splicing, there are two different caspase-2 mRNA isoforms, one encoding caspase-2L and one encoding caspase-2S (Jin et al., 2002). In present study, caspase-2L mRNA levels were determined. Overexpression of caspase-2L induces cell death, while overexpression of caspase-2S suppresses cell death (Ito et al., 2000). This specificity also supports the assumption that the decreased expression of caspase-2 may contribute to protecting organotypic cultures from cell death.

Caspase-3 and caspase-6 belong to the effector caspases (Chong et al., 2005). Caspase-3 has been described as one of the key mediators of the mitochondrial events of apoptosis (Lakhani et al., 2006; Zou et al., 1997). Caspase-6 is highly homologue to caspase-3 but shows different substrate specificity. Expression of both genes is decreased, likely contributing to the survival of cells (MacLachlan and el Deiry, 2002). This observation is in agreement with findings that the transcription and translation of these caspases increase prior to apoptosis (Ginham et al., 2001). Caspase-6 is not only an effector but also a direct inducer of apoptosis without the activation of other caspase effectors in some forms of neuronal death (Zhang et al., 2000). In particular, caspase-6 was shown to be a key proapoptotic enzyme involved in the degradation of nuclear matrix proteins and in activation of caspase-3 activated in renal ischemia (Singh et al., 2002). The inflammatory mediator caspase-1 is significantly expressed in
contribute to cell death (Tamada et al., 2005; Yokota et al., 2005).

Previous studies reported that ischemia and hypoxia generate ROS with 12-20 times higher than those of calpain-1 (Tamada et al., 2005).流行性出血热 is observed in retina, where calpain-2 mRNA levels is twelve times higher than those of calpain-1 (Tamada et al., 2005). The expression pattern is different from the one observed in retina, where calpain-2 mRNA levels is twelve times higher than those of calpain-1 (Tamada et al., 2005). The expression pattern is different from the one observed in retina, where calpain-2 mRNA levels is twelve times higher than those of calpain-1 (Tamada et al., 2005). The expression pattern is different from the one observed in retina, where calpain-2 mRNA levels is twelve times higher than those of calpain-1 (Tamada et al., 2005).

In MOD, the most characteristic changes that we have seen were the transcriptional upregulation of antioxidative SOD-2 (mitochondrial, Mn–SOD) and SOD-3 (extracellular SOD-3), with no changes in SOD-1 (cytosolic, CuZn–SOD). Several previous studies verified that increased SOD activity increases cell survival (Nicotera et al., 2004; Zelko et al., 2002). Recently, a chimeric recombinant superoxide dismutase SOD-2-3 was found to have a protective effect because of its anti-inflammatory properties (Hernandez-Saavedra et al., 2005). We assume that the SOD-2,-3 activity is transcriptionally regulated and the increase of SOD activity would confer protection against damage of the modiolus during the preparation for culture. A wide variety of stimuli including cytokines can induce transcription of SOD-2 and SOD-3 (Zelko et al., 2002). Our complete microarray data accessible on the internet (http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE5446) show that the transcriptional expression of several cytokines and chemokines dramatically increases during culture. Preparing organotypic cultures induce cellular stress combined with the generation of oxidative damage and activation of various cytokines (Dhar-Mascarenos et al., 2005). The expression of cytoplasmic SOD-1 is robust and stable in the inner ear cells of newborn rats.

In addition to the changes in SOD-2 and SOD-3 expression, the Bcl-xl and the caspase-6 expression decreased and Bax expression increased in normoxic and hypoxic cultures. Bax is one of the major proapoptotic family members and its role in hypoxia-induced cell death was recently studied (Greijer and van der, 2004). In cells treated with the strong apoptosis inducer staurosporine, translocation of the proapoptotic protein Bax to the mitochondria was suppressed during hypoxia. Remarkably, cells deficient in Bax and Bak do not undergo cell death during anoxia (Shroff et al., 2007). Several models demonstrated that the ratio of Bcl-2:Bax heterodimers and of the Bax homodimers balance cell survival and cell death (Jin et al., 2001). Here, the increase of Bax U49729 and the decrease of Bcl-xl may contribute to the moderate cell death observed in MOD.

3.2.2. Modiolus

Oxidative stress mediated by ROS is a well recognized damaging factor. A significant source of ROS after anoxic insults are mitochondria (Arundine et al., 2004). The generation of ROS may result in mitochondrial dysfunction and cytochrome c release (Chung et al., 2005). In the MOD, the most characteristic changes that we have seen were the transcriptional upregulation of antioxidative SOD-2 (mitochondrial, Mn–SOD) and SOD-3 (extracellular SOD-3), with no changes in SOD-1 (cytosolic, CuZn–SOD). Several previous studies verified that increased SOD activity increases cell survival (Nicotera et al., 2004; Zelko et al., 2002). Recently, a chimeric recombinant superoxide dismutase SOD-2-3 was found to have a protective effect because of its anti-inflammatory properties (Hernandez-Saavedra et al., 2005). We assume that the SOD-2,-3 activity is transcriptionally regulated and the increase of SOD activity would confer protection against damage of the modiolus during the preparation for culture. A wide variety of stimuli including cytokines can induce transcription of SOD-2 and SOD-3 (Zelko et al., 2002). Our complete microarray data accessible on the internet (http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE5446) show that the transcriptional expression of several cytokines and chemokines dramatically increases during culture. Preparing organotypic cultures induce cellular stress combined with the generation of oxidative damage and activation of various cytokines (Dhar-Mascarenos et al., 2005). The expression of cytoplasmic SOD-1 is robust and stable in the inner ear cells of newborn rats.

In addition to the changes in SOD-2 and SOD-3 expression, the Bcl-xl and the caspase-6 expression decreased and Bax U49729 increased in normoxic and hypoxic cultures. Bax is one of the major proapoptotic family members and its role in hypoxia-induced cell death was recently studied (Greijer and van der, 2004). In cells treated with the strong apoptosis inducer staurosporine, translocation of the proapoptotic protein Bax to the mitochondria was suppressed during hypoxia. Remarkably, cells deficient in Bax and Bak do not undergo cell death during anoxia (Shroff et al., 2007). Several models demonstrated that the ratio of Bcl-2:Bax heterodimers and of the Bax homodimers balance cell survival and cell death (Jin et al., 2001). Here, the increase of Bax U49729 and the decrease of Bcl-xl may contribute to the moderate cell death observed in MOD.

3.2.3. Stria vascularis

The exprssional changes in SV induced during normoxic culture show similarities to changes observed in OC (decrease of caspases and calpain-1) and in MOD (increase of SOD-2). In contrast to MOD, we found decreased expression of SOD-3.

### Table 2 - Fold changes in gene expression observed in normoxic and hypoxia exposed cultures analyzed by microarray and real-time RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Organ of Corti</th>
<th>Modiolus</th>
<th>Stria vascularis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu–No</td>
<td>Cu–Hy</td>
<td>Cu–No</td>
</tr>
<tr>
<td>Sod-1</td>
<td>1.0 (0.7)</td>
<td>1.1 (1.0)</td>
<td>1.3 (1.1)</td>
</tr>
<tr>
<td>Sod-2</td>
<td>7.5 (1.1)</td>
<td>5.3 (1.5)</td>
<td>14* (2.9)</td>
</tr>
<tr>
<td>Sod-3</td>
<td>0.6 (0.7)</td>
<td>0.5 (0.9)</td>
<td>2.6* (2.0)</td>
</tr>
<tr>
<td>Bax</td>
<td>1.8</td>
<td>2.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Bax</td>
<td>2.7 (0.8)</td>
<td>3.2 (0.8)</td>
<td>4.4 (1.4)</td>
</tr>
<tr>
<td>Bax</td>
<td>0.4 (0.9)</td>
<td>0.4 (1.1)</td>
<td>0.4 (1.4)</td>
</tr>
<tr>
<td>Bcl2l1</td>
<td>0.8</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Bcl2l1</td>
<td>2.8 (0.8)</td>
<td>1.4 (0.8)</td>
<td>4.4* (0.7)</td>
</tr>
<tr>
<td>Casp1</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Casp2</td>
<td>0.3* (0.5)</td>
<td>0.3* (0.5)</td>
<td>0.2* (0.6)</td>
</tr>
<tr>
<td>Casp3</td>
<td>1.6 (0.7)</td>
<td>1.8 (0.8)</td>
<td>8.0 (1.3)</td>
</tr>
<tr>
<td>Casp6</td>
<td>0.6 (0.6)</td>
<td>0.2* (0.8)</td>
<td>0.6 (0.7)</td>
</tr>
<tr>
<td>Capn1</td>
<td>0.5 (0.6)</td>
<td>0.5 (0.6)</td>
<td>0.5 (1.0)</td>
</tr>
<tr>
<td>Capn2</td>
<td>1.0</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>p53</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Prnp</td>
<td>0.9 (0.7)</td>
<td>0.9 (0.8)</td>
<td>0.7 (0.8)</td>
</tr>
</tbody>
</table>

Numbers indicate the fold changes calculated from the microarray data and from the real-time RT-PCR data (in brackets: mean values). Cu–No: samples from normoxic organotypic cultures; Cu–Hy: samples from hypoxic organotypic cultures exposed to 5-h hypoxia. Samples were collected for RNA preparation 24 h after onset of culture preparation (Cu–No and Cu–Hy).

* Statistically significant, at least p < 0.05.
mRNA in SV. At present, it is not clear whether this decrease indicates protective or damaging effects. On one hand, SOD-3 enzymatic activity protects the extracellular environment from oxidative stress and preserves nitric oxide bioactivity by scavenging superoxide (Nozik-Grayck et al., 2005). On the other hand, the decreased level of extracellular SOD-3 associates with hypoxia-induced erythropoietin gene expression described by Zelko and Folz (2005). In that report, SOD-3 knock-out mice had a marked increase in hypoxia-induced erythropoietin gene expression, which was more than a 100-fold higher than that in wild-type controls. Recently, we observed that recombinant human erythropoietin protects hair cells from ischemia-induced death (Andreeva et al., 2006).

Another observation was that normoxic culture induced decrease of the Bcl2-like1 (Bcl-xL) gene expression. Generally, the Bcl-xL inhibits apoptosis by neutralizing proapoptotic Bcl-2 family proteins, thus balancing cell death or survival (reviewed in Jin et al., 2001; Soane and Fiskum, 2005). In experimental brain injuries of adult rats, Bcl-xL mRNA level decreased and tended to remain depressed for at least 3 days after injury (Strauss et al., 2004). Surprisingly, in a stretch-injury-induced neural cell death model, Bcl-xL did not provide protection against cell death (Pfister et al., 2004). Here, the decrease in Bcl-xL transcription could be related to the moderate cell death observed in SV (Supplementary Figs. 3 and 4).

### 3.3. Effects of hypoxia on gene expression

In general, hypoxic cultures show similar changes in gene expression as normoxic cultures thus indicating that culture preparation is the dominating factor modifying the gene expression pattern. Remarkably, only the mitochondrial SOD-2

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**Fig. 3** – Relative changes in the gene expression of SOD-1, SOD-2 and SOD-3 in normoxic and hypoxic cultures of OC, MOD and SV. The data are represented by mean values ± SEM, n=6. OC-Co samples were used as a reference. SOD-1: no significant changes were seen. SOD-2: *p<0.001 versus Co; #MOD p<0.001 versus No; *SV p<0.05 versus No. SOD-3: #p<0.001 versus control of OC and MOD (Scheffé’s test); MOD p<0.001 versus Co, when cultured samples were combined (No+Hy; LSD test). *SV p<0.001 versus Co, when cultured samples were combined (No+Hy; LSD test).

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**Fig. 4** – Relative changes in the gene expression of Bax (U49729), Bax (U59184), and Bcl2-like-1 in normoxic and hypoxic cultures of OC, MOD and SV. The data are represented by mean values ± SEM, n=6. OC-Co samples were used as a reference. Bcl2l1: *p<0.005 versus SV-Co. #p<0.01 versus OC-Co and MOD-Co (Scheffé’s test).
responded in a different way to hypoxia as compared to normoxia. After hypoxic culture, SOD-2 mRNA levels increased in the MOD but decreased in the SV, as compared to normoxia. The hypoxia-induced expression modulation of the SOD-2 gene might have resulted from a cytokine signaling (see our full results posted on http://www.ncbi.nlm.nih.gov/geo/) or the combined effect of preparatory and hypoxic stress. This result underlines the importance of ROS in the hypoxia induced cell death.

3.4. Genes without changes in expression

From the genes analyzed here, SOD-1, the cellular prion protein (Prnp), and p53 showed no expression changes, neither under normoxic nor under hypoxic conditions. Interestingly, the steady-state expression level of these genes was relatively high suggesting their important role in vivo and under normoxic or hypoxic culture conditions. For example, SOD-1 mutations are closely associated with the neurodegeneration of motor neurons (Kirby et al., 2002). Moreover, overexpression of SOD-1 protects macrophages against nitric oxide cytotoxicity (Brockhaus and Brune, 1999). Prnp is highly expressed in the brain and, as shown here on a transcript level, in the inner ear. Evidence is accumulating that the normal function of Prnp in neurons is one of neuroprotection (Roucou et al., 2005; Spudich et al., 2005). The response of mRNA levels to damage appears to be tissue-specific. During hippocampal apoptosis after traumatic brain damage, Prnp mRNA levels increased in some brain regions and in others stayed at the same level (Marciano et al., 2004). The high expression level of Prnp in MOD as compared to that in OC and SV points to its special but still unknown role in neurons of the spiral ganglion. P53 protein is an important tumor suppressor and inducer of apoptotic cell death in response to malignant growth (Vousden and Lu, 2002). In the inner ear, p53 protein accumulation was found during age-related hearing loss in CD/1 mice associated with ROS formation and HIF transcription factor upregulation (Riva et al., 2006). Recently, it was shown that the control of p53 over a cell death during hypoxia is exerted via post-translational modification (Lee et al., 2007). This could possibly explain the lack of p53 transcriptional modification in our model.

Fig. 5 – Relative changes in caspase-2, -3, -6 and calpain-1 gene expression in normoxic and hypoxic cultures of OC, MOD and SV using real-time RT-PCR. The data are represented by mean values ± SEM, n = 6. OC-Co samples were used as reference. Caspase-2: *p < 0.005 versus controls (OC and SV) and p < 0.05 versus controls (MOD), using LSD test; caspase-3: *p < 0.05 versus controls, when No and Hy samples were combined using LSD test. Caspase-6: *p < 0.004 versus OC-Co. #p < 0.001 versus OC-Co and SV-Co. SV p < 0.001 versus Co (Scheffé’s test); calpain-1: *p < 0.001 versus the corresponding Co sample (Scheffé’s test).

Fig. 6 – Relative changes in the Prnp gene expression in normoxic and hypoxic cultures of OC, MOD and SV. The data are represented by mean values ± SEM, n = 6. OC-Co samples were used as a reference. *p < 0.001 versus all other Co regions (Scheffé’s test).
3.5. Conclusions

Our results demonstrate that in the inner ear, there are quantitative differences in the transcription of apoptosis-related genes between fresh and cultured tissues. Moreover, 24-h normoxic culture of cochlear structures induced a region-specific differential gene expression, which may be interpreted as a tissue type-specific reaction to a stress-induced cell injury. Overall, in the OC, the in vitro cell survival was associated with transcriptional downregulation of proapoptotic caspases-2, -3, -6 and calpain-1. Hair cells (OHC and IHC) restricted to OC survived well the culture period. In the MOD, the cells survival was associated with the transcriptional upregulation of the anti-apoptotic SOD-2. In the SV, cell survival in normoxic culture was associated with the increase of SOD-2 and the decrease of SOD-3, caspasas and calpain-1. The expression of mRNA encoding the cytoplasmic SOD-1, Prnp, and p53 was robust and stable in all cochlear structures.

In general, hypoxic culture conditions induced the same changes in gene expression as the normoxic conditions indicating that culture preparation is likely the dominating factor, which modifies the gene expression pattern. Hypoxia increased the expression of SOD-2 in the MOD even more than normoxia, implicating possibly important role of the mitochondrial SOD-2 in the spiral ganglion cell survival.

It can be concluded that various culture conditions induce different expression pattern of apoptosis-related genes in the organotypic cochlear cultures, as compared to fresh tissues. This transcriptional pattern may reflect the survival ability of specific tissues and could become a tempting target for a pharmacological intervention in the selected inner ear diseases.

4. Experimental procedure

4.1. Explant cultures

The membranous cochlea from 3- to 5-day-old Wistar rats were prepared. Each cochlea was dissected into the OC, MOD and SV (Sobkowicz et al., 1993). Dissection of the cochlea was performed in buffered saline glucose solution (BSG; 116 mM NaCl, 27.2 mM Na2HPO4, 6.1 mM KH2PO4, glucose 11.4 mM) at 4 °C under a laminar flow hood using a dissecting microscope (Stemi, SV6, Zeiss Germany). The spiral ganglion cell body was prepared in such a way that it remained with the modiolus. We dissected the OC away from the edge of the spiral ganglion, between the limbus and the spiral ganglion throughout the cochlear spiral. The modiolus was cut into two halves to reduce the tissue thickness. The cochlear parts were well preserved for up to 24 h in culture (see supplementary data). For culture, the fragments were incubated in 4-well tissue culture dishes (1.9-cm² culture surface per well, Nunc, Wiesbaden, Germany) in 500 μl Dulbecco's modified Eagle medium/F12 nutrient (1:1) mixtures (DMEM/F12, Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 50 mM glucose, insulin–transferrin–Na–Selenit-Mix 2 μl/ml (Roche Diagnostics GmbH, Mannheim, Germany), penicillin 100 U/ml (Grüenthal GmbH, Aachen, Germany) at 37 °C and 5% CO2 in a humidified tissue culture incubator (Cheng et al., 1999; Lowenheim et al., 1999).

To characterize the viability of the explants we used several criteria: (1) The LDH release assay is a biochemical method to analyze the overall viability of cultures. It measures activity of LDH released from the cytosol of damaged cells into the supernatant (Gramsbergen et al., 2002; Noraberg et al., 1999). An aliquot of culture medium was collected at the start of the culture and 24 h after seeding. In addition, the LDH activity was measured in the tissue of the fragments (Supplementary Fig. 1). (2) Neurofilament (NF) 200 kDa can be used to distinguish neurons from satellite cells in spiral ganglion (Endo et al., 2005; Mou et al., 1997b). A downregulation of (NF200) mRNA is a common feature of human neurodegenerative diseases (Julien and Mushynski, 1998). We analyzed the transcriptional expression of NF200 to characterize the damage of the neuronal structures (Supplementary Fig. 2). (3) To analyze the viability of the cultures on a cellular basis we used propidium iodide (PI; 1 μg/ml; Molecular Probes, Eugene, Oregon, USA), and calcein AM (10 μM, Molecular Probes Europe, Leiden, The Netherlands) (Noraberg et al., 1999) (Supplementary Fig. 3). PI enters dead or dying cells via damaged cell membranes and interacts with DNA to yield a brightly red fluorescence. Calcein AM permeates free into cells and is cleaved by intracellular esterases of living cells producing a green fluorescence (Ripoll and Rebillard, 1997). (4) To measure cell viability by visualizing healthy and damaged nuclei, we used Hoechst dye 33342 (SIGMA) combined with PI, as described previously (Ciancio et al., 1988) followed by confocal microscopy analyses (Supplementary Fig. 4). (5) In the OC, inner and outer hair cells (IHC, OHC) were counted along the organ of Corti, immediately after preparation and after the 24-h culture period. Hair-cell staining and counting were carried out as recently described (Mazurek et al., 2003). All studies were performed in accordance with the German Prevention of Cruelty to Animals Act and permission was obtained from the Berlin Senate Office for Health (T0234/00).

4.2. Hypoxia

Three hours after plating, the cultures were exposed to hypoxia at 37 °C for 5 h in an incubation chamber (Billups-Rothenberg, Del Mar, CA, USA) as described elsewhere (Gao et al., 1999). In brief, the chamber with plates was perfused with a calibrated gas mixture of 5% CO2, 95% N2 (AGA Gas GmbH, Bottrop, FRG) for 15 min. The oxygen pressure inside the culture medium was 15–20 mm Hg after 10-min exposure time and reached a steady-state level of 10–20 mm Hg after 25 min up to the end of hypoxia. After hypoxia, the cultures were kept under normoxic conditions for another 16 h. Organotypic cultures subjected to temporary hypoxic conditions are referred in text to as "hypoxic" cultures or conditions. Thus, both normoxic and hypoxic explant cultures were washed and suspended in a lysis buffer (Qiagen, Hilden, Germany) for RNA isolation 24 h after preparation of the culture.

4.3. RNA isolation and quantification

Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) strictly according to the manufacturer's protocol. The RNA content was determined with the RiboGreen RNA
RNA of OC, MOD and SV from 3 animals were pooled to obtain total RNA each. The RNA samples originated from three independent series of RNA preparations within 1 year: Series 1: Samples OC1, OC2, MOD, SV from freshly prepared tissue of 3- to 5-day-old rats indicated as controls (Co). Series 2: Samples originated from culture of OC, MOD and SV under normoxic (Cu-No) and hypoxic conditions (Cu-Hy). Series 3 was a repetition of Series 2.

Altogether 16 independent RNA samples and 4 arrays were used for the microarray study. For the RT-PCR study, the total RNA isolated from OC, MOD and SV from 3 animals were pooled to obtain one sample.

4.4. cDNA microarray analysis

The quality of the RNA used for the microarray was analyzed by an Agilent 2100 Bioanalyzer (Agilent Technology, Palo Alto, CA, USA). The microarray analysis was carried out as recently described (Chaitidis et al., 2005). Briefly, from total RNA, double-stranded cDNA was synthesized using T7 (dT)24 primers containing a T7 RNA polymerase promoter sequence. To produce biotin-labeled cRNA from the cDNA, an in vitro transcription was carried out. The quality and quantity of each RNA/DNA sample were assessed by gel electrophoresis and spectrophotometric analysis. Labeled and processed cRNA samples (6 μg) were hybridized to the Affymetrix Rat Neurobiology U34 Array (RN-U34; 1322 gene transcripts, Affymetrix, Santa Clara, USA). After washing and staining of the arrays in an Affymetrix-Fluidics station, the arrays were scanned on an Agilent scanner. Hybridization was performed by the Laboratory for Functional Genome Research, Charité-University Medicine Berlin (UU, RJK). All processes were subjected to the QC protocols as described in the Affymetrix Gene Chip Manual. Raw data quantitation kit (Molecular Probes, Göttingen, Germany). For the microarray study, the total RNA isolated from OC, MOD and SV of 6 animals were pooled to obtain a sample of 7.5 μg total RNA each. The RNA samples originated from culture of OC, MOD and SV under normoxic (Cu-No) and hypoxic conditions (Cu-Hy). Series 3 was a repetition of Series 2.

4.6. RT-PCR

Primers were designed using the primer designer (Scientific and Educational Software, version 3.0). In general, designs were based on sequences indicated on the Affymetrix website (http://www.affymetrix.com). PCR primers were custom synthesized by BIOTEZ (Berlin, Germany). Primers used for RT-PCR (Acc. No., forward, reverse, product length):

- SOD-1 (M21060), 5′-aac atg gcc gtc cag cag at-3; 5′-gcc aag cgg ctt cca gca tt-3′ (226);
- SOD-2 (Y00497) 5′-gcc acc tac tgt gag aat ct-3′, 5′-ttg ata gcc tcc agc aac tc-3′ (200);
- SOD-3 (Z24721) 5′-agg cga gca gaa cac ctc ca-3′, 5′-cca cga agt tgc cga agt cc-3′ (143);
- Bax (U49729) 5′-cca aga agc tga gcg aag gt-3′, 5′-tca cgg agg aag tcc tgt gt-3′ (271);
- Bax (US9184) 5′-cgt ggt gca gct gac ttt-3′, 5′-agt gga gac tcc agc cac aa-3′ (267);
- Bcl2 (U34963) 5′-acc tga atg acc acc tag ag-3′, 5′-act tcc gac tga aga tgt ag-3′ (183);
- Casp2 (U77933), 5′-aca ggt gat tgt ggt gtg tc-3′, 5′-gga ggt cag cca tca agt ag-3′ (105);
- Casp3 (U49930), 5′-ata cca gta ggc gac gac ttt-3′, 5′-cag ctt gtc cgc gta cag tt-3′ (132);
- Casp6 (AF025670), 5′-ggt tct tct gcc gcc acc tag ca-3′, 5′-gaa gct tct tgg cta ggt gt-3′ (122);
- Calp1 (U53859), 5′-tcg ctc gcc gcc gga gtt cca cc-3′, 5′-gca ctc gat ggc ctc tgt gtt cc-3′ (218);
- Pmp (D50093), 5′-cag ccc cgg ccc gct gag ctc-3′, 5′-cga ccc cct cca tca tc-3′ (142).

Reverse transcription (RT) was done as follows: Step 1: 5.6 μl H2O, 1 μl (10 μM) 3′-primer, 5 μl RNA (30–150 ng/μl), 0.4 μl RNasin Ribonuclease Inhibitor (Promega; 40 μl/μl, Promega); 5 min at 70 °C, cooling to 4 °C. Step 2: Addition of a 8.37 μl master mix containing 4 μl M-MLV Reverse Transcriptase buffer (5×), 0.125 μl M-MLV Reverse Transcriptase 200 μl/μl, 0.25 μl RNasin Ribonuclease Inhibitor (Promega), 4 μl 2.5 mM dNTP Mix, PCR Grade (invitrogen). Step 3: Reverse transcription was performed for 60 min at 42 °C, 5 min at 95 °C, and cooling to 4 °C.

Real-time PCR was performed using the LightCycler FastStart DNA MasterPLUS SYBR Green 1 (Roche Diagnostics GmbH, Penzberg, Germany) in the Light Cycler System (Roche Diagnostics, Basel, Switzerland). 15 μl of master mix was filled in the glass capillaries (9.25 μl water, 0.75 μl 3′-primer, 10 μM, 1 μl 5′-primer, 10 μM, 4 μl LightCycler FastStart DNA MasterPLUS SYBR Green 1, Roche). Then, 5 μl of cDNA was added as PCR template. After centrifugation, capillaries were placed inside the cycler. A typical settings for targets of 100–280 bp in length included the following steps: (1) preincubation at 95 °C for 10 min; (2) amplification (35–40 cycles) including denaturation at 95 °C for 10 s, annealing at 66 °C for 10 s, extension at 72 °C, 20 s; (3) melting curve program at 60–95 °C with a heating rate of 0.1 °C/s; (4) Cooling down to 40 °C. To reveal the variability of the method, we calibrated each run using as a standard a pool of different cochlear regions. The day to day variation coefficient over all genes varied between 2.1 and 27.3 (mean of VC=8.1±7.6, n=40).
4.7. Quantification of mRNA

Determination of gene expression level by real-time RT-PCR was carried out by a standard curve method for relative quantification of the different samples and the comparative CP method for differential gene expression (Pfaffl, 2001; Pfaffl et al., 2002). The efficiency of the amplification was estimated using serial dilutions of RNA samples. Optimal thermal cycling conditions were tested for each gene separately. The real-time RT-PCR efficiency was calculated from the slope according to the equation: $E = 10^{(-1/slope)}$. Three criteria were used for an optimal RT-PCR reaction: (i) efficiency within the range of $E = 1.80$ to $2.00$, (ii) a single band resolved during agarose electrophoresis (single product of the desired length) and (iii) a well-defined melting curve (single product-specific melting temperature). Using optimized conditions, one run always included at least one complete set of experimental samples, i.e., OC-Co, OC-No, OC-Hy; MOD-Co, MOD-No, MOD-Hy; SV-Co, SV-No, SV-Hy and, in addition, aliquots of two reference samples which were used for day to day variation analysis of the RT-PCR data and for the evaluation of the differential gene expression using the comparative CP method (Pfaffl, 2001; Pfaffl et al., 2002).

The relative content of the experimental samples was expressed in arbitrary units (AU) and calculated on the basis of the CP values, the efficiency curves and the RNA mass input. The basic equation describing real-time PCR amplification is

$$ F = N_0 \cdot E^{CP_t} $$

(1)

where $F$ is the fluorescence signal intensity at the crossing point, $N_0$ is the amount, $E_t$ is the amplification efficiency and $CP_t$ is the crossing point of the target molecules at the RNA input level.

The amount of the target can be calculated by Eq. (6) derived from Eq. (1).

$$ N_t = \frac{F}{E_t^{CP_t}} $$

(6)

By setting $F$ equal to one and using CP values normalized to one ng RNA input, arbitrary units (AU) can be calculated by using Eq. (7). To facilitate data handling, the obtained arbitrary units are multiplied by $1 \times 10^{11}$.

$$ AU = \frac{1}{E_t^{CP_t}} $$

(7)

The comparative CP method was used for analysis of differential gene expression (Pfaffl, 2001; Pfaffl et al., 2002). This model incorporates the amplification efficiencies of the target and the reference genes. The target gene expression is normalized by a non-regulated reference gene expression, e.g., derived from the so-called housekeeping genes. In the present paper, we used SOD-1 as a reference, a gene which did not show statistically significant differences among the experimental samples, using microarray and RT-PCR.

$$ r = \frac{\Delta CP_t^{exsa}}{\Delta CP_t^{calisa-exsa}} $$

(8)

Symbols: $r$—expression of target normalized to that of reference gene; $\Delta CP_t^{exsa}$—difference of the CP values of the targets, normalized to one ng RNA input, of the calibrator and experimental samples; $\Delta CP_t^{calisa-exsa}$—difference of the CP values of the reference genes, normalized to one ng RNA input, of the calibrator and experimental samples.

For RT-PCR analyses, we selected (1) genes which showed a differential gene or region specificity in their expression of the control samples and (2) genes which showed unexpected or remarkable changes in their expression during culture and after hypoxia exposure.

4.8. Statistics of RT-PCR

Mean ± standard deviation (SD) or standard errors of the mean (SEM) were calculated of all parameters measured. The significance of the differences between the different regions and treatment groups was assessed using analysis of variance (ANOVA). A significant ANOVA was followed by a post hoc Scheffé’s test. Differences of $p < 0.05$ were considered to be significant. For comparing statistically significant findings between microarray and qRT-PCR, in addition to Scheffé’s post hoc test, the LSD post hoc test was used to indicate tendencies of the result.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2007.05.061.

REFERENCES


