Original Paper

Audiology & Neurotology

Audiol Neurotol 2010;15:282–290
DOI: 10.1159/000279760

Received: April 23, 2009
Accepted after revision: November 20, 2009
Published online: February 3, 2010

Stress and Survival Pathways in the Mammalian Cochlea

Antje Caelers[a] Vesna Radojevic[a] Jens Traenkle[b] Yves Brand[a] Daniel Bodmer[a]

[a]Department of Biomedicine, University Hospital Basel and Klinik für Ohren-, Nasen-, Halskrankheiten, University Hospital Basel, Basel, and [b]Zeptosens – a Division of Bayer AG, Witterswil, Switzerland

Key Words
Protein kinase Akt · Aminoglycosides · Gentamicin · Hair cells · NF-κB transcription factor · Apoptosis signaling pathway p38

Abstract
Studies conducted over the last few years demonstrated that signaling pathways that operate in the organs of Corti (OC) play a central role in survival and death of hair cells. An important goal of molecular otology is to characterize these signaling pathways in normal inner ears and inner ears exposed to a variety of different forms of stress, such as ototoxic substances and noise overexposure. In this study, we used high-performance reverse protein microarray technology and phospho-specific antibodies to examine the activation status of defined molecules involved in cellular signaling. We demonstrate that reverse protein microarrays based on the highly sensitive planar-waveguide technology provide an effective and high-throughput means to assess the activation state of key molecules involved in apoptotic and prosurvival signaling in microdissected OC explants over time. In this study, we show that gentamicin and a specific NF-κB inhibitor increase the ratio of phospho-c-Jun/c-Jun in OC explants of postnatal rats soon after exposure to these drugs. In addition, we found a decrease in the phospho-Akt/Akt ratio in OC explants early after NF-κB inhibition. Finally, we observed an early and consistent decrease in the phospho-p38/p38 ratio in OC explants exposed to the NF-κB inhibitor and only a transient decrease in this ratio in OC examples after gentamicin exposure.

Introduction

Hearing loss due to sensorineural damage has been recognized for over a century. Until recently, damage to cochlear hair cells (HCs) and neurons has been regarded as an inevitable consequence of age, genetic conditions or exposure to certain environmental stimuli. During the past several years, however, some of the critical intracellular events that mediate aspects of damage to HCs have been discovered [Cheng et al., 2005; Matsui and Cotanche, 2004]. In a large number of studies, aminoglycosides have been utilized as inducers of HC death, and a significant progress in understanding aminoglycoside-induced HC death has been made [Lautermann et al., 2004; Rybak and Whitworth, 2005]. After aminoglycoside exposure, cellular and biochemical alterations in the HCs of the organ of Corti (OC) are set into motion. Reactive oxygen species have been detected in vitro soon after

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1420–3030/10/0155–0282$26.00/0

Accessible online at:
www.karger.com/aud

Dr. Daniel Bodmer
Klinik für Ohren-, Nasen-, Halskrankheiten, University Hospital Basel
Petersgraben 4
CH–4031 Basel (Switzerland)
Tel. +41 61 328 7603, Fax +41 61 265 4029, E-Mail dbodmer@uhbs.ch

exposure to aminoglycosides [Priuska and Schacht, 1995; Sha et al., 2001; Choung et al., 2009]. In addition, it has been demonstrated that small GTPases, such as Ras and Rho/Rac/Cdc42, as well as the c-Jun-N-terminal kinase (JNK) signaling pathway, are activated in cells exposed to the drug [Battaglia et al., 2003; Bodmer et al., 2002a, b; Pirvola et al., 2000; Jiang et al., 2006a]. JNK activation results in phosphorylation of c-Jun and in activation of the transcription factor activating protein-1 complex [Albinger et al., 2006; Matsui et al., 2004]. Finally, caspases are activated and HCs undergo apoptotic cell death after prolonged aminoglycoside exposure [Huang et al., 2000; Okuda et al., 2005]. However, chronic administration of aminoglycosides induces also caspase-independent pathways of HC death [Jiang et al., 2006b].

Interestingly, gentamicin exposure also activates pathways that promote HC survival [Battaglia et al., 2003]. This is in agreement with the current opinion that cells are thought to exist in a finely tuned balance between survival and cell death [Boatright and Salvesen, 2003; Raff, 1992]. According to this concept, exposure to cellular stress disrupts this balance and if the stress is severe, apoptosis-promoting pathways predominate and the cells die.

Lately, survival pathways which operate in HCs have been defined. One such pathway is the H-Ras/Raf/MEK/Erk pathway [Battaglia et al., 2003]. Another pathway is the phosphatidylinositol-3 kinase (PI3k) pathway. In an interesting publication by Chung et al. [2006], it has been shown that PI3k mediates HC survival and opposes gentamicin toxicity in neonatal rat OC explants via its downstream target, the protein kinase Akt. This finding is in good agreement with data from our laboratory. We demonstrated recently that the transcription factor NF-κB is required for survival of immature auditory HC in vitro [Nagy et al., 2005, 2007] in a PI3k-dependent pathway. In addition, in an interesting publication by Schacht and colleagues, it has been shown that the aminoglycoside kanamycin stimulates the activation of NF-κB and that this activation protects cochlear HCs from aminoglycoside-induced ototoxicity [Jiang et al., 2005].

Reversible protein phosphorylation is one of the most abundant posttranslational modifications. Protein kinases transfer a phosphate group from adenosine triphosphate to serine, threonine or tyrosine residues. This process is reversible, and dephosphorylation occurs by enzymes called phosphatases. Protein kinases and protein phosphatases are regulated by extracellular stimuli, and the activity of these enzymes leads to dynamic changes in protein phosphorylation and subsequently determines whether a signal transduction pathway is turned on or switched off. In order to gain insight into the molecular mechanisms involved in these processes, the phosphorylation status of key molecules involved in HC damage/death and survival need to be investigated. In this study, we used highly sensitive reverse protein microarrays which allow generating high-fidelity data for protein expression profiling of low levels or transient states of protein abundance [Pawlak et al., 2002; Weissenstein et al., 2006; Oostrum and Voshol, 2008]. We wanted to apply this new technology, namely planar-waveguide reverse protein microarrays, to the characterization and direct monitoring of the activity and protein phosphorylation of key molecules involved in apoptotic and prosurvival signaling in the OC. We hoped that the high sensitivity of the method would allow the parallel analysis of the phosphorylation status of several selected signaling marker proteins in single microdissected OC explants.

During the last decades, ELISAs have been used to measure the expression level of a defined single protein. However, ELISA applications are often laborious, and time- and material-consuming. In contrast, protein arrays have the potential to analyze a multiple set of proteins simultaneously in only minute amounts of a biological sample. Protein arrays are mostly based on translating the well-established single-analyte ELISA assay format into a miniaturized, multiplexed assay combined with chemiluminescence or fluorescence readout [Templin et al., 2002]. In order to achieve adequate sensitivity for the signal detection, various amplification schemes have been developed. Zeptosens has developed a microarray system based on planar waveguide technology featuring an advanced surface-confined fluorescence signal generation and read-out scheme providing very high sensitivity while using less reagent and sample material compared to other microarray systems (www.zeptosens.com) [Pawlak et al., 2002]. While the inner ear offers only tiny amounts of tissue for analysis, we tested this protein array system for its potential use in inner ear research.

For the analysis, we chose two members of apoptosis signaling pathways (c-Jun and p38) and one member of a survival pathway (Akt). We investigated the ratios of the cellular levels of phosphorylated versus nonphosphorylated forms of the transcription factor c-Jun and of the protein kinase Akt over time in OC cultures exposed to either gentamicin or to an NF-κB inhibitory peptide. In addition, we analyzed the expression levels of phosphorylated versus nonphosphorylated forms of p38, a protein kinase involved in apoptosis and cell survival pathways.

Material and Methods

Tissue Culture

All animal procedures were carried out according to an approved animal research protocol (Kantonales Veterinaeramt, Basel, Switzerland). For tissue culture, 5- to 6-day-old Wistar rat pups were used. The rats were killed, and cochlear microdissections were performed under a light microscope to isolate the OC, spiral ganglion, and stria vascularis [Sobkowicz et al., 1993]. After isolation, all OCs were transferred to cell culture plates and maintained on 0.4-mm culture plate inserts (Millipore) in Dulbecco Modified Eagle Medium with 25 M HEPES supplemented with 10% fetal calf serum (all Invitrogen) and 30 U/ml penicillin (Sigma).

Gentamicin Time Course Experiment

OCs were obtained as described above. They were maintained for 48 h in culture medium for recovery. OCs were then exposed to gentamicin at the concentration of 0.5 mM for 2, 4, 6, 9, and 15 h. We recently demonstrated that this concentration of gentamicin results in significant HC loss after 15 h [Caelers et al., 2009]. Control explants were maintained in culture medium alone for 0 and 15 h. For each time point, 1 OC was studied. Each experiment was repeated three times for both conditions (culture medium alone or gentamicin treatment). After culturing, OC explants were lysed in 40 μl of CLB96 buffer (Zeptosens).

NF-κB Inhibition Time Course Experiment

To inhibit NF-κB activity, an HPLC-purified synthetic inhibitor (NF-κB inhibitor, AAVALLPAVLLALLAPVQRKRQKLMP, Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) was used at the final concentration of 25 μg/ml in culture medium. We have previously shown that this compound at this concentration causes HC loss within several hours [Nagy et al., 2005]. OCs were obtained as described above. They were maintained for 48 h in culture medium for recovery. OCs were then exposed to the NF-κB inhibitor at the concentration of 25 μg/ml for 2, 6, and 15 h. Control explants were maintained in culture medium alone for 0 and 15 h. For each time point, 1 OC was studied. Each experiment was repeated three times for both conditions (culture medium alone or NF-κB inhibitor treatment). After culturing, OC explants were lysed in 40 μl of CLB96 buffer (Zeptosens).

Microarray Printing, Blocking, and Analysis

Microarrays were produced employing a standard protocol as described in detail [Oostrum et al., 2009]. Briefly, total protein lysates were spotted in serial fourfold dilutions on ZeptoMARK hydrophobic chips (Zeptosens) as droplets of 400 pl in duplicate with a noncontact piezoelectric microdispenser (Nanoplotter 2.0, GeSiM). Chips were dried for 1 h at 37 °C and were blocked with blocking buffer BB1 for 20 min using the ZeptoFOG ultrasonic nebulizer (Zeptosens). After blocking, the chips were washed with water and spin-dried in a swinging-bucket centrifuge with an appropriate holder (available from Zeptosens) at 200 *g* for 3 min. The chips were stored at 4 °C in the dark until use in reverse protein microarray assays. Each of the three biological replicates (independently treated OC explants) was analyzed in two independent microarray experiments.

For relative quantification of the immobilized proteins, chips were blocked with 1% Tween-80 in water following a similar protocol as described above. After the washing steps and drying, the chips were incubated with filtered Sypro Ruby Protein Dye (Invitrogen), diluted 1:5 in water for 30 min and were washed again three times. Relative amounts of immobilized total protein lysates were quantified using the green detection channel (λex/λem = 532/570 nm) of a ZeptoREADER (Zeptosens).

BB1-blocked chips were used for high-performance protein microarrays analysis. The chips were incubated with antibodies to phospho-(Thr308)-Akt, Akt, phospho-(Ser73)-c-Jun, c-Jun, phospho-(Thr180, Tyr182)-p38 MAPK and p38 MAPK in assay buffer CAB1 (Zeptosens) overnight. Then, after rinsing the chips with CAB1 three times, an incubation step with corresponding fluorophore-labeled anti-species antibodies (Alexa647-labeled rabbit or mouse Fab, Invitrogen Z25308 or Z25108, respectively) followed. After another wash cycle, protein phosphorylation was assessed by measuring fluorescence intensities in the red channel (λex/λem = 635/670 nm) of a ZeptoREADER (Zeptosens). Images were analyzed with the ZeptoVIEW pro image processing software (version 2.0, Zeptosens), and relative fluorescence intensities were calculated by plotting of net spot intensities relative to protein concentration as described [Pawlak et al., 2002; Oostrum et al., 2009] and normalized to the relative amount of immobilized protein. Errors were calculated with Gauss' error progression.

ELISA Assay

An ELISA assay was used to quantify total Akt and phospho-Akt according to the manufacturer's protocol (R&D Systems). Briefly, all OC explants were maintained for 24 h in culture medium. Further, OC explants were cultured in fresh culture medium alone for 6 or 15 h (control), or were exposed to the NF-κB inhibitor at the concentration of 25 μg/ml for 6 or 15 h. The explants were then used for ELISA analysis to determine phospho-Akt and Akt in the two groups. The data were collected from four independent experiments and statistically analyzed (Student's t test).

Western Blot (Antibody Validation)

The following phospho-specific antibodies were tested for their selectivity for the phosphorylated epitope: phospho-(Thr308)-Akt, phospho-(Ser73)-c-Jun, phospho-(Thr180, Tyr182)-p38 MAPK. Antibodies were tested for specificity at a 1:1000 dilution by Western Blotting as described [Towbin et al., 1979] using whole cell extracts from untreated, calyculin-treated (induction of Akt phosphorylation), UV-irradiated (induction of c-Jun phosphorylation) or anisomycin-treated (induction of p38 phosphorylation) HeLa cells according to a protocol by Zeptosens.

Results

Gentamicin Exposure or Exposure of OC Explants to the NF-κB Inhibitor Results in an Increase in the Phospho-c-Jun/c-Jun Ratio

A time course analysis of the phospho-c-Jun to total c-Jun protein ratio reveals a significant increase in OC explants exposed to 0.5 mM gentamicin for 6 h (fig. 1a) or 2 h of the NF-κB inhibitor at the concentration of 25 μg/

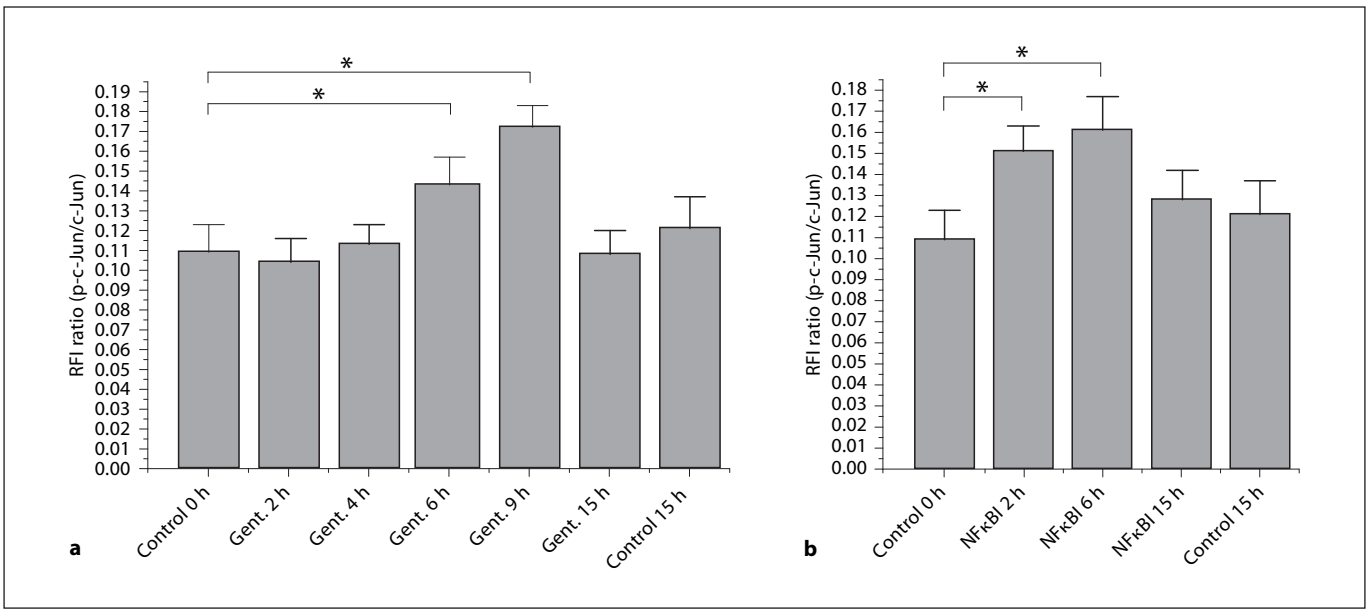

Fig. 1. Activation profile of c-Jun in OC explants represented as phospho-(Ser73)-c-Jun vs. total c-Jun protein ratio. OC explants were treated with gentamicin (**a**) or the NF-κB inhibitor (NFκBI; **b**) for the indicated times before lysis and subsequent microarray analysis. Each bar in the diagram represents the mean of three biological replicates (independently treated OC explants), which were analyzed in two independent microarray experiments. Asterisks indicate a statistically significant ($p < 0.05$) difference between the indicated groups. Errors are calculated with a Gauss error progression. Histograms represent mean + standard deviation. RFI = Relative fluorescence intensity.

ml (fig. 1b), as compared to control explants. The peak ratio can be observed after 9 h of gentamicin exposure and 6 h of exposure to the NF-κB inhibitor. Cell culturing itself does not change the phospho-c-Jun/c-Jun ratio, as we observe no difference when OC explants are held in culture medium alone (0 and 15 h).

Exposure of OC Explants to the NF-κB Inhibitor Results in a Decrease in the Phospho-Akt/Akt Ratio and Gentamicin Exposure Does Not Change the Phospho-Akt/Akt Ratio over Time

A time course analysis of the phospho-Akt to total Akt protein ratio reveals a decrease in the phospho-Akt/Akt ratio in OC explants when exposed to the NF-κB inhibitor for 2 and 6 h (fig. 2a), with a minimum of the phospho-Akt/Akt ratio after 6 h of exposure as compared to control explants. An independent ELISA experiment confirmed the decrease in the phospho-Akt/Akt ratio in OC explants exposed to the NF-κB inhibitor for 6 h as compared to control explants maintained in culture medium alone for 6 h (fig. 3). Exposure of OC explants to gentamicin does not change significantly the phospho-Akt/Akt ratio over time as revealed by the analysis of the array data (fig. 2b) or the ELISA analysis (fig. 4). Cell culturing itself does not change the phospho-Akt/Akt ratio, as we observe no difference when OC explants are held in culture medium alone (0 and 15 h).

Gentamicin Exposure or Exposure of OC Explants to the NF-κB Inhibitor Results in an Early Decrease in the Phospho-p38/p38 Ratio

A time course analysis of the phospho-p38 to total p38 protein ratio reveals a significant decrease in the phospho-p38/p38 ratio in OC explants exposed to 0.5 mM gentamicin for 4 h (fig. 5a) or the NF-κB inhibitor at the concentration of 25 μg/ml for 2, 6, and 15 h (fig. 5b), as compared to control explants. The decrease in the ratio occurs early after gentamicin exposure (minimum after 4 h) or exposure to the NF-κB inhibitor (minimum after 6 h). Interestingly, the phospho-p38/p38 ratio increases after the initial drop in the gentamicin-treated samples, while the samples treated with the NF-κB inhibitor display a permanent decrease in the ratio over time. Cell culturing itself also changes the phospho-p38/p38 ratio, as we observe a decrease in the ratio when OC explants are held in culture medium alone for differing lengths (0 and 15 h).

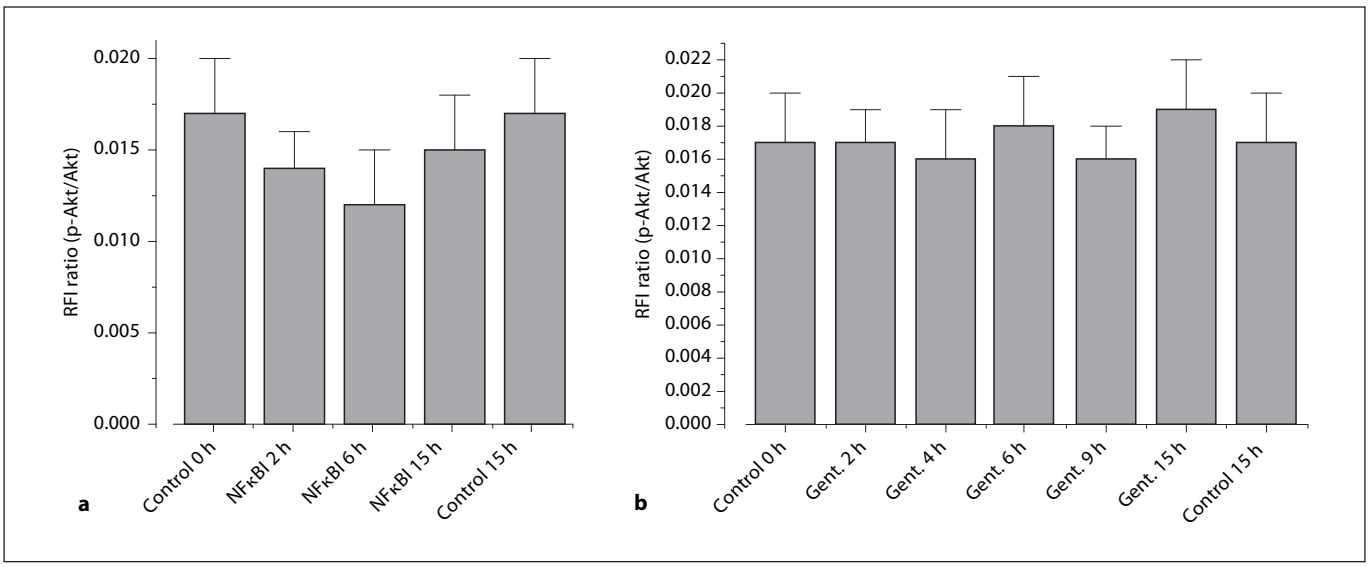

Fig. 2. Activation profile of Akt in OC explants represented as phospho-(Thr308)-Akt vs. total Akt protein ratio. OC explants were treated with the NFκBI (**a**) or gentamicin (**b**) for the indicated times before lysis and subsequent microarray analysis. Each bar in the diagram represents the mean of three biological replicates (independently treated OC explants), which were analyzed in two independent microarray experiments. Asterisks indicate a statistically significant ($p < 0.05$) difference between the indicated groups. Errors are calculated with a Gauss error progression. Histograms represent mean + standard deviation.

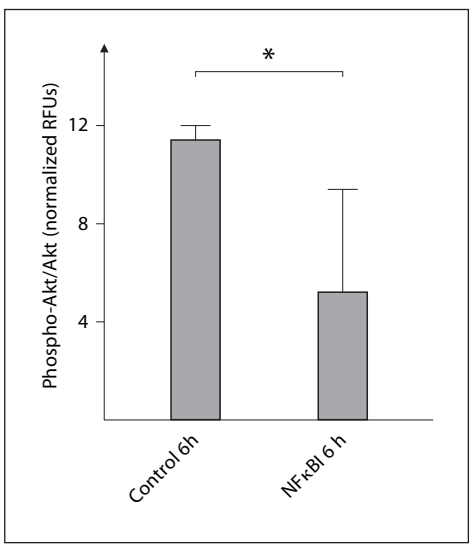

Fig. 3. Phospho-Akt/Akt ratio as determined by ELISA analysis. OC explants were held in culture medium alone or treated with the NFκBI for the indicated time before ELISA analysis. Each bar in the diagram represents the mean of five biological replicates (independently treated OC explants) + standard deviation. The decrease in the phospho-Akt/Akt ratio after NF-κB inhibition was statistically significant ($p < 0.05$).

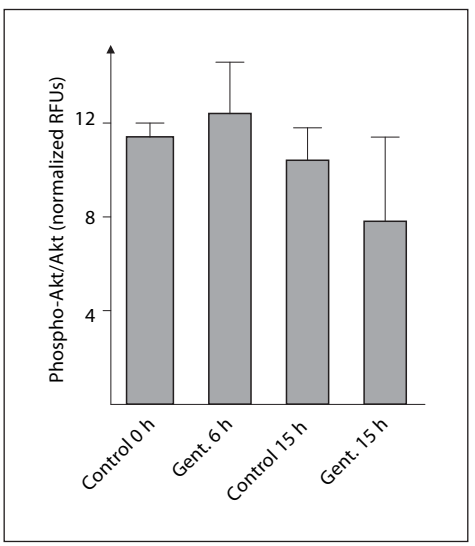

Fig. 4. Phospho-Akt/Akt ratio as determined by ELISA analysis. OC explants were held in culture medium alone or treated with gentamicin for the indicated time before ELISA analysis. Each bar in the diagram represents the mean of four biological replicates (independently treated OC explants) + standard deviation. The changes in the phospho-Akt/Akt ratio in the different groups were not statistically significant.

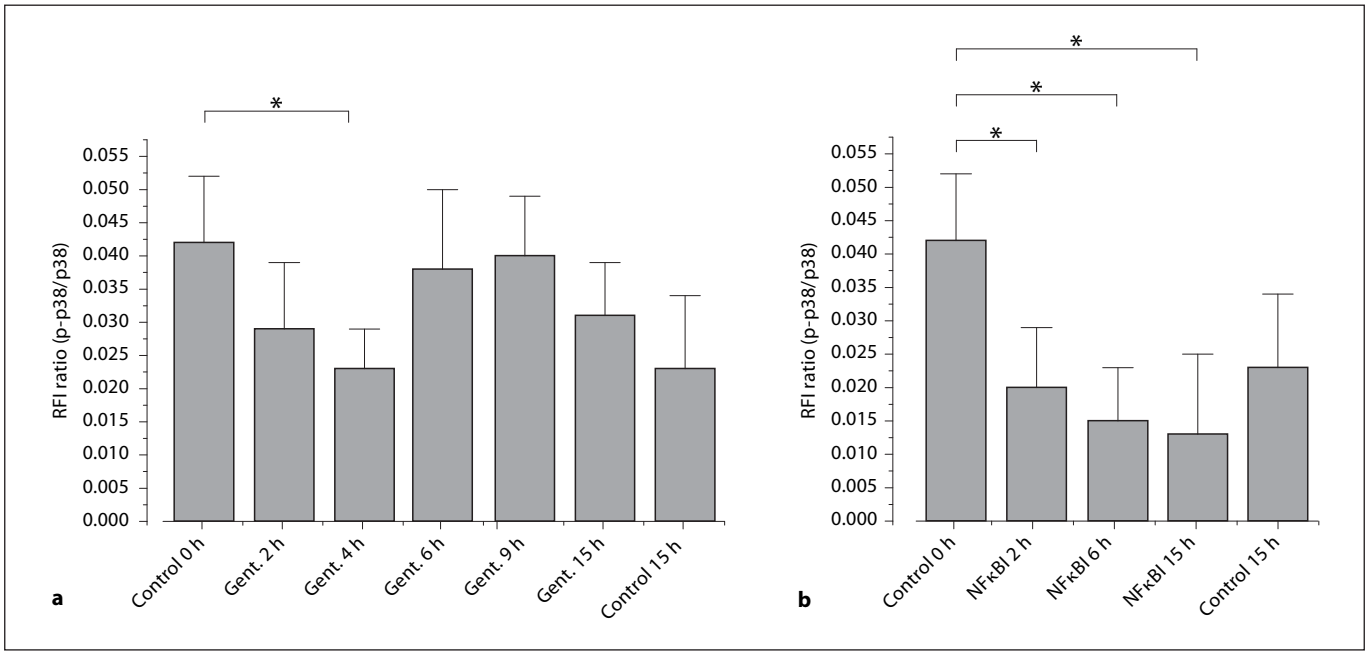

Fig. 5. Activation profile of p38 in OC explants represented as phospho-(Thr180, Tyr182)-p38 MAPK vs. total p38 MAPK protein ratio. OC explants were treated with gentamicin (**a**) or the NFκBI (**b**) for the indicated times before lysis and subsequent microarray analysis. Each bar in the diagram represents the mean of three biological replicates (independently treated OC explants), which were analyzed in two independent microarray experiments. Asterisks indicate a statistically significant ($p < 0.05$) difference between the indicated groups. Errors are calculated with a Gauss error progression. Histograms represent mean + standard deviation.

Anti-Phospho Antibodies Used Detect a Single Species in Western Blots

Western blot analysis revealed that the antibodies used in this study (antibodies against phospho-c-Jun, phospho-Akt, phospho-p38) detect just a single species (fig. 6).

Discussion

The goal of this study was to investigate whether the planar-waveguide reverse protein microarrays can be used as a tool to analyze signal transduction events upon damage in the OC. The advantage of this new technology is that it is highly sensitive, so that a single OC can be probed with multiple antibodies. Therefore, precious material can be saved and fewer animals have to be sacrificed. A further advantage of this method is that even transient modifications of proteins, such as phosphoproteins, can be analyzed. However, the analysis of phosphoproteins is based on phospho-specific antibodies that are sometimes insensitive to cross-reactivity since the sequence around the phosphorylated residue is highly conserved. To demonstrate that the phospho-specific antibodies used in this study are specific, we used Western blots to show that the antibodies used detect a single species (fig. 6). In the literature so far, several pathways have been implicated in damage to the OC. Here, we have used this new method to study some of these pathways. It should be noted that our in vitro data presented here are limited to newborn rat pups, because only OCs from newborn animals can be used for tissue culture. However, the immature cochlea is an established model for the study of aminoglycoside toxicity [Pirvola et al., 2000]. Still, in vitro toxicity assessed in neonatal tissue may occur by different mechanisms than in vivo toxicity in adults. As an example, it has been demonstrated that high-dose aminoglycoside treatment in vitro can lead to rapid blebbing of the apical HC surface [Richardson and Russel, 1991]. This phenomenon has not been observed in vivo. Therefore, we used relatively low gentamicin dosages (0.5 mM) to mimic features of adult in vivo toxicity.

In line with the literature, the data of the present study demonstrate that the JNK pathway is involved in HC loss

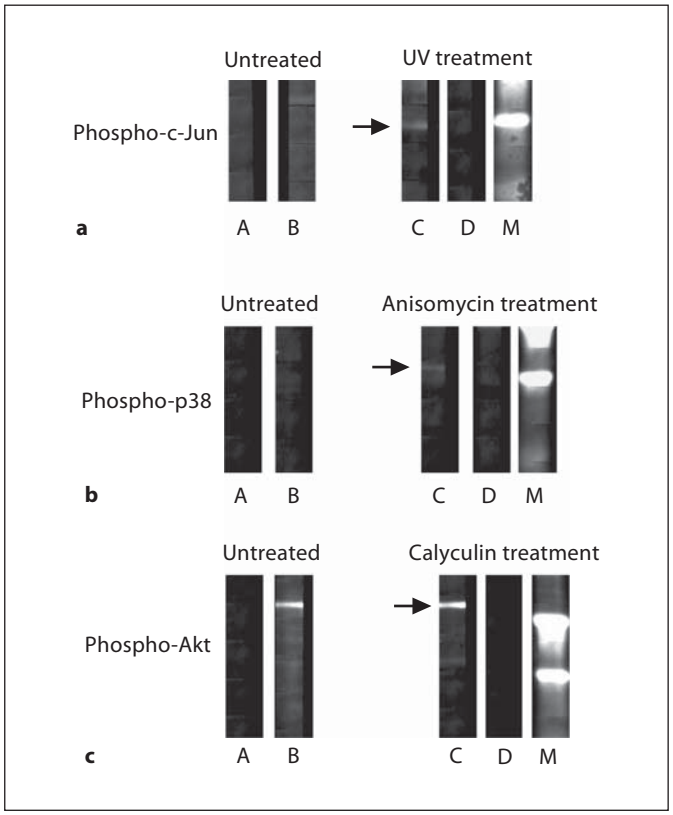

Fig. 6. a Western blot analysis demonstrates the presence of a single species (arrow) detected by the antibody against phospho-c-Jun in HeLa cells after UV treatment (lane C). Untreated HeLa cells with the secondary antibody only (lane A), untreated HeLa cells with primary and secondary antibody (lane B), and treated HeLa cells with the secondary antibody only (lane D) do not show this band. M = Molecular marker. **b** Western blot analysis demonstrates the presence of a single species (arrow) detected by the antibody against phospho-p38 in HeLa cells after anisomycin treatment (lane C). Untreated HeLa cells with the secondary antibody only (lane A), untreated HeLa cells with primary and secondary antibody (lane B), and treated HeLa cells with the secondary antibody only (lane D) do not show this band. **c** Western blot analysis demonstrates the presence of a single species (arrow) detected by the antibody against phospho-Akt in untreated HeLa cells (lane B) and after calyculin treatment (lane C). Untreated HeLa cells with the secondary antibody only (lane A) and treated HeLa cells with the secondary antibody only (lane D) do not show this band.

due to aminoglycoside gentamicin. Since JNK activation results in phosphorylation of c-Jun in HC nuclei [Bodmer et al., 2002a, b; Pirvola et al., 2000], we used this pathway as a reference to test whether our array data would demonstrate the same findings. We show that the phosphorylated c-Jun to c-Jun ratio increases in OC explants exposed to 0.5 mM gentamicin for 6 and 9 h (fig. 1). This observation is in good agreement with the literature [Matsui and Cotanche, 2004; Pirvola et al., 2000]. We observed a peak of phosphorylated c-Jun/c-Jun in OC explants after 9 h of exposure to gentamicin. This fits well with our previous observation that phosphorylated c-Jun can be seen in outer and inner HCs of the OC 12 h after gentamicin exposure using immunohistochemistry [Bodmer et al., 2002a]. Cell culturing of OC explants in medium alone does not seem to activate the JNK signaling pathway, as we observed the same ratio of phospho-c-Jun/c-Jun after culturing OC explants for 0 and 15 h without gentamicin treatment (fig. 1a). NF-κB inhibition also led to activation of the JNK signaling pathway: we observed an increase in phosphorylated c-Jun/c-Jun in OC explants after 6 h of NF-κB inhibition as compared to control OC explants maintained in culture medium alone (fig. 1b). Taken together, our results concerning the JNK pathway are in good agreement with the literature, and therefore suggest that this new technology is a powerful tool for the analysis of signaling events in cultured OC explants.

We have previously shown that NF-κB inhibition results in rapid HC loss from cultured OC explants [Nagy et al., 2005]. In a follow-up study using gene arrays and a biochemical assay, we were able to link the NF-κB-induced HC loss to PI3k [Nagy et al., 2007]. Interestingly, the PI3k pathway has been previously linked to HC survival [Chung et al., 2006] and to aminoglycosides [Schacht et al., 1977; Jiang et al., 2006c]. Activated PI3k phosphorylates the membrane lipid PIP2 at the 3′-OH position to generate PIP3. PIP3 recruits signaling proteins such as Akt and PDK-1. The activation state of Akt is an indicator of the state of the PI3k survival pathway. In this study, we observed an early decrease in the phospho-Akt/Akt ratio in OC explants exposed to an NF-κB inhibitor as compared to control OC explants maintained in cell culture medium alone (fig. 2a). Using an ELISA analysis, we were able to confirm the protein microarray data: OC explants exposed to the NF-κB inhibitor also demonstrated a decrease in the phospho-Akt/Akt ratio as compared to control explants maintained in culture medium alone for the same time period (6 h; fig. 3). The decrease was more significant with ELISA than it was with the array. It might be that this quantitative difference in the phospho-Akt/Akt ratio using the two methods is due to the cross-reactivity that might have occurred using the ELISA kit or due to the possibility that ELISA might have been somewhat more sensitive in this particular case. In contrast to the situation in OC explants treated with the NF-κB in-

hibitor, we did not observe a difference in the ratio of phospho-Akt/Akt in OC explants treated with gentamicin (fig. 2b and 4). Chung et al. [2006] observed a discrete increase in phospho-Akt after gentamicin exposure, as revealed by Western blot analysis. However, that study did not assess the phospho-Akt to Akt ratio and a different gentamicin concentration (0.05 vs. 0.5 mM gentamicin) was used. Taken together, previous reports and data from this study implicate that NF-κB promotes HC survival via its downstream targets PI3k and Akt.

Further on, we observed a significant decrease in the phospho-p38 to total p38 ratio in OC explants exposed either to gentamicin (fig. 5a) or to the NF-κB inhibitor (fig. 5b). However, this decrease was only transient after exposure to gentamicin. Wei et al. [2005] reported an increase in p38 phosphorylation after exposure of OC explants to gentamicin as revealed by immunohistochemistry. At first look, this report seems to contradict our finding. However, the ratio of phospho-p38 to total p38 in the control at 15 h in our experiment is also much lower compared to the control at 0 h, it is also lower than the ratio at 15 h after gentamicin exposure. Since Wei et al. [2005] have performed the experiment at 16 h, it might be extrapolated from our graph in figure 5a, assuming that there is a continuous drop in the ratio of phospho-p38 to total p38 in the control explants over the whole time course of 15 h, that at that time point there was an increase in p38 phosphorylation in the explants exposed to gentamicin when compared to the control explants. It seems that phospho-p38 is very unstable as its level is very much reduced in the control at 15 h. Still, the drop is even more significant at 4 h after gentamicin exposure. It is hard to interpret the presented data, but perhaps it might be concluded that as the immediate response to gentamicin, phospho-p38 is dephosphorylated and the longer exposure to gentamicin triggers its phosphorylation.

The decrease in phospho-p38/p38 after exposure to the NF-κB inhibitor was more significant and was measured at all time points, indicating that the NF-κB inhibitor induces dephosphorylation of phospho-p38.

Overall, this study shows that the new technology of reverse phase protein microarray can be very useful for analyzing the signal transduction pathways in the damaged OC. The presented data confirm some of the published findings and reveal new ones. However, one has to keep in mind that this new method, as any other method used so far for the analysis of events in the OC, is limited by the fact that numerous cell types in the OC make it difficult to discern differences in cell type-specific expression. Since our analysis is based on whole OC cultures, our data reflect protein level and phosphorylation changes from all the cells that compose the OC, among which are HCs, but also various supporting cells. However, we know from previous studies that the signaling pathways described in this article operate in HCs of the OC. Our group demonstrated that gentamicin treatment results in increased phospho-c-Jun staining of HC nuclei and no staining of supporting cell could be observed [Bodmer et al., 2002a, b]. Likewise, it has been shown that gentamicin induces phosphorylation of p38 in HCs and not in supporting cells of the OC [Wei et al., 2005]. Finally, aminoglycoside exposure of mice in vivo results in decreased phospho-Akt staining of HCs, whereas no staining could be observed in the supporting cells of the OC [Jiang et al., 2006c].

This method can prove useful for other purposes as well, as besides analyzing intracellular events involved in damage of HCs in the OC, it might be used for profiling of cytokine expression during inflammation of the inner ear or for detecting proteins which are expressed in low levels in inner ear tissue.

References

Albinger-Hegyi A, Hegyi I, Nagy I, Bodmer M, Schmid S, Bodmer D: Alteration of activator protein-1 DNA binding activity in gentamicin-induced hair cell degeneration. Neuroscience 2006;137:971–980.

Battaglia A, Pak K, Brors D, Bodmer D, Frangos J, Ryan AF: Involvement of ras activation in toxic hair cell damage of the mammalian cochlea. Neuroscience 2003;122:1025–1035.

Boatright KM, Salvesen GS: Caspase activation. Biochem Soc Symp 2003;70:233–242.

Bodmer D, Brors D, Bodmer M, Ryan A: Rescue of auditory hair cells from ototoxicity by CEP-11004, an inhibitor of the JNK signalling pathway. Laryngorhinootologie 2002b;81:853–856.

Bodmer D, Brors D, Pak K, Gloddek B, Ryan A: Rescue of auditory hair cells from aminoglycoside toxicity by *Clostridium difficile* toxin B, an inhibitor of the small GTPases Rho/Rac/Cdc42. Hear Res 2002a;172:81–86.

Caelers A, Monge A, Brand Y, Bodmer D: Somatostatin and gentamicin-induced auditory hair cell loss. Laryngoscope 2009;119:933–937.

Cheng AG, Cunningham LL, Rubel EW: Mechanisms of hair cell death and protection. Curr Opin Otolaryngol Head Neck Surg 2005;13:343–348.

Choung YH, Taura A, Pak K, Choi SJ, Masuda M, Ryan AF: Generation of highly-reactive oxygen species is closely related to hair cell damage in rat organ of Corti treated with gentamicin. Neuroscience 2009;161:214–226.

Chung WH, Pak K, Lin B, Webster N, Ryan AF: A PI3k pathway mediates hair cell survival and opposes gentamicin toxicity in neonatal rat organ of Corti explants. JARO 2006;7: 373–382.

Huang T, Chen AG, Stupak H, Liu W, Kim A, Staecker H, Lefebvre PP, Malgrange B, Kopke R, Moonen G, Van de Water TR: Oxidative stress-induced apoptosis of cochlear sensory cells: otoprotective strategies. Int J Dev Neurosci 2000;18:259–270.

Jiang H, Sha SH, Forge A, Schacht J: Caspase-independent pathways of hair cell death induced by kanamycin in vivo. Cell Death Differ 2006b;13:20–30.

Jiang H, Sha SH, Schacht J: The NF-kappaB pathway protects cochlear hair cells from aminoglycoside-induced ototoxicity. J Neurosci Res 2005;79:644–651.

Jiang H, Sha SH, Schacht J: Rac/Rho regulates actin depolymerization induced by aminoglycoside antibiotics. J Neurosci Res 2006a;83:1544–1551.

Jiang H, Sha SH, Schacht J: Kanamycin alters cytoplasmic and nuclear phosphoinositide signalling in the organ of Corti in vivo. J Neurochem 2006c;99:269–276.

Lautermann J, Dehne N, Schacht J, Jahnke K: Aminoglycoside- and cisplatin-ototoxicity: from basic science to clinics. Laryngorhinootologie 2004;83:317–323.

Matsui JI, Cotanche DA: Sensory hair cell death and regeneration: two halves of the same equation. Curr Opin Otolaryngol Head Neck Surg 2004;12:418–425.

Matsui JI, Gale JE, Warchol ME: Critical signalling events during the aminoglycoside-induced death of sensory hair cells in vitro. J Neurobiol 2004;61;250–266.

Nagy I, Caelers A, Monge A, Bonari S, Huber AM, Bodmer D: NF-kappaB-dependent apoptotic hair cell death in the auditory system. Audiol Neurotol 2007;12:209–220.

Nagy I, Monge A, Albinger-Hegyi A, Schmid S, Bodmer D: NF-kappaB is required for survival of immature auditory hair cells in vitro. JARO 2005;6:260–268.

Okuda T, Sugahara K, Takemoto T, Shimogori H, Yamashita H: Inhibition of caspases alleviates gentamicin-induced cochlear damage in guinea pigs. Auris Nasus Larynx 2005;32: 33–37.

Oostrum J, Calonder C, Rechsteiner D, Ehrat M, Mestan J, Fabbro D, Voshol H: Tracing pathway activities with kinase inhibitors and reverse phase protein arrays. Proteomics 2009; 3:412–422.

Oostrum J, Voshol H : Antibody-based proteomics to study cellular signalling networks. Eur Pharm Rev 2008;2:31–35.

Pawlak M, Schick E, Bopp MA, Schneider MJ, Oroszlan P, Ehrat M: Zeptosens' protein microarrays: a novel high performance microarray platform for low abundance protein analysis. Proteomics 2002;2:383–393.

Pirvola U, Xing-Qun L, Virkkala J, Saarma M, Murakata C, Camoratto AM, Walton KM, Ylikkoski J: Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. J Neurosci 2000;20:43–50.

Priuska EM, Schacht J: Formation of free radicals by gentamicin and iron and evidence for an iron/gentamicin complex. Biochem Pharmacol 1995;50:1749–1752.

Raff MC: Social controls on cell survival and cell death. Nature 1992;356:397–400.

Richardson GP, Russell IJ: Cochlear cultures as a model system for studying aminoglycoside-induced ototoxicity. Hear Res 1991;53: 293–311.

Rybak LP, Whitworth CA: Ototoxicity: therapeutic opportunities. Drug Discov Today 2005;10:1313–1321.

Schacht J, Lodhi S, Weiner ND: Effects of neomycin on poly-phosphoinositides in inner ear tissue and monomolecular films. Adv Exp Med Biol 1977;84:191–208.

Sha SH, Taylor R, Forge A, Schacht J: Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. Hear Res 2001;155:1–8.

Sobkowicz HM, Loftus JM, Slapnick SM: Tissue culture of the organ of Corti. Acta Otolaryngol Suppl 1993;502:3–36.

Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gel sto nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4354.

Tremplin MF, Stoll D, Schrenk M, Traub PC, Vöhringer CF, Joos TO: Protein microarray technology. Trends Biotechnol 2002;20:160–166.

Wei X, Zhao L, Dodel RC, Farlow MR, Du Y: Minocycline prevents gentamicin-induced ototoxicity by inhibiting p38 MAP kinase phosphorylation and caspase-3 activation. Neuroscience 2005;131:513–521.

Weissenstein U, Schneider MJ, Pawlak M, Cicenas J, Eppenberger-Castori S, Oroszlan P, Ehret S, Geurts-Moespot A, Sweep FCGJ, Eppenberger U: Protein chip based miniaturized assay for the simultaneous quantitative monitoring of cancer biomarkers in tissue extracts. Proteomics 2006;6:1427–1436.