Non-small cell lung cancer cell survival crucially depends on functional insulin receptors

Carolin Maria Frisch, Katrin Zimmermann, Pia Zilleßen1, Alexander Pfeifer, Kurt Racké and Peter Mayer1
Institute of Pharmacology and Toxicology, University of Bonn, Sigmund-Freud-Straße 25, 53127 Bonn, Germany
1Federal Institute for Drugs and Medical Devices (BfArM), Kurt-Georg-Kiesinger-Allee 3, 53175 Bonn, Germany

Abstract
Insulin plays an important role as a growth factor and its contribution to tumor proliferation is intensely discussed. It acts via the cognate insulin receptor (IR) but can also activate the IGF1 receptor (IGF1R). Apart from increasing proliferation, insulin might have additional effects in lung cancer. Therefore, we investigated insulin action and effects of IR knockdown (KD) in three (NCI-H292, NCI-H226 and NCI-H460) independent non-small cell lung cancer (NSCLC) cell lines. All lung cancer lines studied were found to express IR, albeit with marked differences in the ratio of the two variants IR-A and IR-B. Insulin activated the classical signaling pathway with IR autophosphorylation and Akt phosphorylation. Moreover, activation of MAPK was observed in H292 cells, accompanied by enhanced proliferation. Lentiviral shRNA IR KD caused strong decrease in survival of all three lines, indicating that the effects of insulin in lung cancer go beyond enhancing proliferation. Unspecific effects were ruled out by employing further shRNAs and different insulin-responsive cells (human pre-adipocytes) for comparison. Caspase assays demonstrated that IR KD strongly induced apoptosis in these lung cancer cells, providing the physiological basis of the rapid cell loss. In search for the underlying mechanism, we analyzed alterations in the gene expression profile in response to IR KD. A strong induction of certain cytokines (e.g. IL20 and tumour necrosis factor) became obvious and it turned out that these cytokines trigger apoptosis in the NSCLC cells tested. This indicates a novel role of IR in tumor cell survival via suppression of pro-apoptotic cytokines.

Key Words
- apoptosis
- carcinoma
- IGF receptor
- insulin
- insulin receptor

Introduction
Cancer and type 2 diabetes mellitus (T2DM) are among the most frequent chronic diseases worldwide with a rapidly increasing number of people affected (Vigneri et al. 2009, Rahati et al. 2014). Various epidemiological studies reveal that T2DM is positively associated with tumorigenesis (Li & Kong 2014). In the population of diabetic patients an increased incidence of liver (El-Serag et al. 2006), pancreatic (Everhart & Wright 1995), breast (Wolf et al. 2005), colorectal (Larsson et al. 2005), endometrial (Friberg et al. 2007), bladder (Larsson et al. 2006) and kidney cancer, (Bao et al. 2013) as well as elevated rates of cancer mortality have been observed (Barone et al. 2008, Vigneri et al. 2009).

Many tumor cells express insulin as well as insulin-like growth factor1 (IGF1) receptors (IGF1R). According to the current state of knowledge, insulin-binding to the IGF1R, known to trigger mitogenic intracellular pathways, is a
substantial reason for insulin’s cancer-promoting effects (Cohen 2006, Warnken et al. 2010). Relevant structural and functional similarities of the peptides insulin and IGF1, and their receptors, are responsible for this cross-reaction binding (De Meyts & Whittaker 2002). In this respect, certain insulin analogues (insulin molecules with modified amino acid sequence to achieve more favorable pharmacokinetic properties) have been critically discussed because of increased affinity to IGF1Rs (Shukla et al. 2009). However, recent studies give evidence that the insulin receptor (IR) itself plays a crucial role in malignancies (Frasca et al. 2008). Importantly, Kim et al. (2012) further revealed for the first time the impact of IR in survival of non-small cell lung cancer (NSCLC) cells. This is particularly important because of the recent development of insulin for inhalation in order to avoid the need of frequent s.c. injections.

Several studies confirm that inhaled insulin is a reasonable alternative to injection for type 2 diabetics (Cappelleri et al. 2002, Royle et al. 2003, Santos Cavaiola & Edelman 2014). EXUBERA (Pfizer) was the first inhalation delivery system, but it was withdrawn in 2007 because of a cumbersome inhaler (Heinemann 2008). Since June 2014, a new inhalable insulin with an improved inhaler, AFREZZA (MannKind Corporation, Valencia, CA, USA), is approved by the FDA and was recently launched in the USA. Despite these technological advances, concerns about a safe, lifelong therapy with inhalable insulin should not be underestimated (Royle et al. 2004). Effects of supra-physiological levels of this anabolic hormone in the respiratory system bear a potential risk of promoting cell proliferation.

In this study, we therefore aimed to illuminate the roles of insulin and the IR in different NSCLC cell lines in more detail, to draw conclusions on pharmacological safety aspects in the therapy with inhalable insulin and, more generally, to obtain deeper insight in the role of the IR in tumor progression.

Materials and methods

Cell lines and cell culture

NSCLC cell lines NCI-H292 (mucoepidermoid pulmonary carcinoma), NCI-H226 (metastatic squamous cell carcinoma) and NCI-H460 (large cell lung carcinoma) were obtained directly from American Type Culture Collection (ATCC) (Manassas, VA, USA). ATCC had authenticated the cells were used. Cells were cultured in RPMI 1640 medium (GE Healthcare, Little Chalfont, Buckinghamshire, UK) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% v/v fetal bovine serum (PBS; Biochrom, Berlin, Germany) and incubated in a humidified incubator at 37 °C and 5% CO2.

[^3H]-thymidine incorporation

After trypsinization, cells were seeded in 12-well dishes with a density of 1×10⁵ cells/well and incubated for 24 h in culture medium. Subsequently, cells were cultured for 24 h in serum-free RPMI medium, before test compounds insulin or IGF1 were added for 29 h. During the last 24 h of substance incubation, [^3H]-thymidine (37 MBq/ml, PerkinElmer, Waltham, MA, USA) was present in the medium. This period was followed by cell preparation to detect radioactivity incorporated into DNA (Freitag et al. 1996, Matthiesen et al. 2006). Cells were washed twice with PBS (4 °C), lysed with 5% w/v trichloroacetic acid for 10 min, again washed with PBS (4 °C) and treated with 0.1 M NaOH for 1 h at 37 °C to extract DNA from cell lysates. After neutralization of the alkaline cell solution with Tris–HCl, scintillation cocktail (Lumac LSC, Groningen, The Netherlands) was added to each sample and radioactivity was recorded by liquid scintillation spectrometry (Packard 2100 TR liquid scintillation analyzer).

Real-time qPCR analysis

Quantitative PCR (qPCR) was performed with SYBR Green in a StepOnePlus Real-Time PCR System (Applied Biosystems). Amounts of mRNA expression are presented as relative quantification by normalizing crossing point values of target genes to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The nucleotide sequences of the PCR primers used are listed in Table 1. For all primer pairs an annealing temperature of 72 °C was used.

Western blot analysis

After dissemination of 3×10⁵ cells/six-well dish and incubation for 24 h, culture medium was removed gently, and cells were washed twice with ice-cold PBS. Cellular proteins were extracted in RIPA buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% w/v sodium deoxycholate, 1% v/v Nonidet P-40, 0.1% w/v SDS, 2 mM EDTA), supplemented with protease inhibitors PMSF (1 mM), pepstatin A (0.7 μg/ml), and leupeptin (0.5 μg/ml). Then 15 μg of total protein/sample was mixed in a dilution of 3:1 with reducing protein loading buffer (Roti-Load 1; Roth, Karlsruhe, Germany).
Germany) and denaturated at 70 °C for 10 min. Whole-cell lysate was separated by SDS–PAGE in NUPAGE-buffer system (Invitrogen) and subsequently transferred (20% v/v methanol, 25 mM Tris, 192 mM glycine) onto PVDF membranes (Millipore, Billerica, MA, USA). In accordance with manufacturer’s protocol, blots were blocked in 5% w/v dried milk protein in tris-buffered saline (150 mM NaCl, 50 mM Tris, 0.1% v/v Tween 20) over night. IR and IGF1R proteins were detected by mouse monoclonal antibody IR (β-subunit) antibody CT-3 (Calbiochem, Bad Soden, Germany) and rabbit polyclonal antibody IGF1R (β-subunit) receptor antibody C-20 (sc-713, Santa Cruz Biotechnology) (Table 2). This was followed by 1 h incubation with peroxidase-conjugated secondary goat antibodies (Bio-Rad) in 5% w/v dried milk protein in tris–buffered saline Tween. Subsequently, treatment with (BM) chemoluminescence blotting substrate peroxidase (POD) (Roche) and exposure to Hyperfilm (Amersham Biosciences) were applied to visualize bands on the membrane. Differing from the general western blot protocol, for detection of phospho-IR and phospho-IGF1R, an incubation time of 24 h in starving medium was included before cells were treated with water (control sample) or insulin in various concentrations for 15 min. Phosphatase inhibitors sodium fluoride (NaF, 35 mM) and sodium orthovanadate (Na3VO4, 1 mM) were added to the lysis buffer to prevent dephosphorylation during protein preparation, SDS–PAGE and blotting. Detection of the target proteins was conducted with the antibodies listed in Table 2. In order to quantify the intensity (in arbitrary units) of the ECL-developed bands, densitometric analysis with ImageJ Software was performed (NIH, Bethesda, MA, USA; http://imagej.nih.gov/ij/). After background subtraction, optical density was normalized to loading control. The relative density values were then calculated and are presented as percentage of the respective experimental controls.

Production of lentiviral vectors and transduction of NSCLC cells

Stable knockdown (KD) of IR was performed by transduction with LVs expressing U6 promoter driven shRNA against IR. By the use of two constructs with a different efficiency in IR KD, effects of a strong KD (LV-sh-IRa: GCTCTGTTACCTTGTACCGCACTAT) were compared to effects of a weak KD (LV-sh-IRb: CCACCATTCGAGTCTGAAGAT). IGF1R KD was performed (LV-sh-IGF1R: GCCTTTCACATTGTACCGCAT) to analyze differences to IR KD effects. As controls pLKO.1-U6-CMV-GFP-PGK-Puro (LV-GFP) and pLKO.1-U6-sh-ctl-PGK-Puro (LV-sh-control) were used. Generation of LVs was carried out as previously described (Pfeifer & Hofmann 2009). In brief, human embryonic kidney cells (HEK 293T cells, ATCC), seeded in DMEM (Gibco, Germany) on Poly-L-lysin coated 150 mm² dishes, served as packaging cells. For LV transduction, 1.5 × 10⁵ cells/six-well dish for protein analysis or 2 × 10⁴ cells/24-well dish for mRNA analysis were seeded and incubated for 4 h until cells adhered. Medium was removed and replaced by 1 ml (for six-wells) or 0.4 ml (for 24-wells) medium containing polybrene (3 μg/ml) and 150 ng (per six-well) or 20 ng RT (per 24-well) LVs. 72 h after

Table 1  Nucleotide sequences of the PCR primers used for amplification of genes of interest

<table>
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<tr>
<th>Abbreviation of gene name</th>
<th>Accession number</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
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<td>GAPDH</td>
<td>NM_002046</td>
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<tr>
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<td>CAAGAGATGATTGAGATGGCCGCA</td>
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<tr>
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<tr>
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<tr>
<td>IGF1R</td>
<td>NM_000875</td>
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<td>CAGAGTATTGAGATGGCCGCA</td>
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<tr>
<td>TNF</td>
<td>NM_000594</td>
<td>CTTCAACGCTAATAAGTGTTG TCAC</td>
<td>GCAGCTGCTGAGGGCCAA</td>
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</table>

*Splice variant sensitive.

Table 2  Source of antibodies used for western blotting and size of target protein

<table>
<thead>
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<th>Protein name</th>
<th>Manufacturer</th>
<th>Cat. No</th>
<th>Molecular Wt (kDa)</th>
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<td>β-actin</td>
<td>Sigma–Aldrich</td>
<td>A3854</td>
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<tr>
<td>Phospho-Akt</td>
<td>Cell Signaling</td>
<td>9271</td>
<td>60</td>
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<tr>
<td>Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)</td>
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<tr>
<td>ERK 2 MAPK</td>
<td>Cell Signaling</td>
<td>9101</td>
<td>44/42</td>
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<tr>
<td>pIGF1Rβ</td>
<td>Santa Cruz</td>
<td>sc-154</td>
<td>42</td>
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<tr>
<td>/pIRβ (Tyr1150/1151)</td>
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<td>3024</td>
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<td>IGF1Rβ</td>
<td>Calbiochem</td>
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<tr>
<td>Irβ</td>
<td>Calbiochem</td>
<td>GR36</td>
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transduction (Day 3 (D3)) cells were used for analysis or virus-mixture was replaced by starving medium and cells were incubated for 24 h (D4) or 48 h (D5).

Cell survival assay

For determination of cell survival, cells were washed twice with PBS and trypsinized 72 h after viral transduction (D3). In order to perform cell count at D4 and D5, cells were treated as described in the transduction section. Cell count was ascertained by measurement with CedEx XS Analyzer (Roche). Results are presented as absolute cell number/ml.

Caspase 3/7 assay

Detection of Caspase 3/7 activity was conducted with 1×10⁴ cells/96-well microplates (Greiner Bio-One, Frickenhausen, Germany) using Caspase-Glo 3/7 Assay (Promega) in accordance to the manufacturer’s protocol. To study effects of cytokines which were upregulated in gene expression profiling (see Results) on apoptosis, cells were incubated for 48 h with 100 nM of each test compound. The chemotherapeutic drug gemcitabine (Cayman, Ann Arbor, MI, USA) was selected as positive control. Samples treated with water served as negative control. Activation levels of caspases in cytokine-stimulated samples are shown as percentage of untreated control levels. In order to elucidate implications of IR KD in programmed cell death, Caspase-Glo 3/7 Assay was performed at D3 after LV transduction. Both LV-sh-control vector-treated and untreated cells were used as negative controls. Caspases activities of IR KD samples are represented as percentage of LV-sh-control cells.

Gene expression profiling

Conduction of DNA chip hybridization was performed as described previously (Mayer et al. 2012). In brief, samples were amplified and labeled using Low RNA Input Linear Amp Kit (Agilent Technologies, Santa Clara, CA, USA). Control samples were labeled with Cy3 and samples from stimulated cells with Cy5. DNA chip hybridization was conducted on Agilent Whole Human Genome Oligo (60-mer) 4×44 K microarrays corresponding to the 60-mer oligo microarray processing protocol of the Gene Expression Hybridization Kit (Agilent Technologies). For statistical analysis, ratios of expression levels in IR KD vs sh-control cells were calculated and subjected to logarithmic transformation (base 2). Induction in gene expression is signified by a positive and suppression by a negative sign.

Results

Insulin and IGF1 receptor expression in NSCLC cells

Conventional PCR (conducted with the same equipment as the qPCR presented below) revealed presence of IR in all three bronchial carcinoma cell lines tested (Fig. 1A). Furthermore, it turned out that both IR splicing variants (IR-A and IR-B) were present in all cells but in a markedly different ratio. The splicing variants are distinguished by the presence or absence of Exon 11. The primer pair used spanned exon 11 and hence produced amplificates of different lengths for the two variants. H292 cells expressed both splicing variants in nearly equal amounts; the longer variant IR-B (+ Ex11), known to trigger metabolic effects of insulin, dominated in H226, whereas H460 predominantly expressed the shorter variant IR-A (−Ex11), which is mainly involved in mitogenic effects of insulin.

Quantitative real-time PCR (qRT-PCR, Fig. 1B, white bars) revealed that IR expression was most pronounced in H292 cells. The expression level in H226 cells was similar (0.8-fold of H292 IR mRNA, difference not statistically significant) whereas in H460 cells, IR mRNA was significantly lower as compared to H292 cells (0.3-fold of H292 IR mRNA).

The amount of IGF1R mRNA (Fig. 1B, black bars) was highest in H292 cells, i.e. 11-fold and sixfold higher as compared to H226 and H460 cell level, respectively. The IGF1R/IR ratio was highest in H292 (10:1) and H460 (5:1) cells. The expression levels of the two receptors hardly differed from each other in H226 cells.

Quantification of western blot analysis with anti-IR monoclonal antibody verified that cell line H292 (assigned to 100%) expressed IR protein most strongly, followed by H226 (76±1%) and H460 (44±3%) (Fig. 1C and D). A high level of IGF1R protein was found in H292; expression in H226 and H460 cells was markedly lower (Fig. 1E and F).

Effects of insulin on cell proliferation and mitogenic signaling

The metabolic effects of insulin are mediated by the activation of the Akt (=protein kinase B) signaling cascade following IR autophosphorylation. Simultaneously, insulin can trigger a mitogenic signaling pathway characterized by phosphorylation of ERK/MAPK. Therefore, we studied activation of these two major insulin signaling pathways in the NSCLC cells and, in parallel, proliferation of the cells measured as [³H]-thymidine incorporation.
In order to cover insulin actions caused by concentrations which are presumably achieved in the respiratory system after inhaling insulin (Mayer et al. 2012), cells were treated with insulin in a range between 1 nM and 1 μM (Fig. 2A). In these concentrations, insulin binds to IR and IGF1R (Vigneri et al. 2010).

In H292 cells, concentration-response curve revealed an increase by 17 ± 3% in $[^{3}H]$-thymidine incorporation by 1 nM and 180 ± 20% by 1 μM (Fig. 2A). This insulin-dependence was observed in H226, too, albeit with a reduced potency and a reduced maximal response (100 ± 21% with 1 μM insulin, Fig. 2A). In H460 cells, insulin did not affect cell proliferation (Fig. 2A), which is in line with the low IR expression level in this cell line (Fig. 1B). For comparison, $[^{3}H]$-thymidine incorporation was also determined after incubation with IGF1 (1–10 nM) (Fig. 2B). Again, H292 cells responded with a significantly increased $[^{3}H]$-thymidine incorporation rate (43 ± 5% for 1 nM and 102 ± 20% for 10 nM IGF1). Similar results were obtained in H460 cells. However, proliferation of H226 cells was not affected by IGF1 (Fig. 2B).

Without external stimulus (basal conditions), H460 turned out to be the most rapidly proliferating cell line with a five- and a tenfold enhanced growth rate as compared to H226 and H292 cells, respectively (Fig. 2C).

Subsequently, we aimed to characterize intracellular signaling pathways following insulin stimulation. We therefore focused on the phosphatidylinositol 3-kinase (PI3K)-Akt and the ERK/MAPK pathways (Taniguchi et al. 2006). Both cascades are linked to proliferation and activated by growth hormones. The first step of IR/IGF1R signaling was tested by analysis of receptor autophosphorylation. Due to the large structural homology of IR and IGF1R at the relevant site, a combined phospho (p)IGF1R/pIR antibody was used. Western blot analysis demonstrated a concentration-dependent stimulation of the tyrosine residues Y1135/1136 (IGF1R) and Y1150/1151 (IR) after exposure to insulin; stimulation with 100 nM led to a significant increase in IR/IGF1R activation in all cell lines tested (Fig. 3A and B). Only in H292 some constitutive activity of the receptors, identified by a (weak) pIGF1R/pIR signal in absence of insulin, was detected (Fig. 3A).

Akt activity under basal conditions (i.e. in absence of insulin) was observed in H226 and H460, but was not detectable in H292 cells (Fig. 3C). Insulin (further) induced Akt-signaling in a concentration-dependent manner in the range of 100 pM to 100 nM in the NSCLC lines used.

Phospho-western blot analysis revealed ERK/MAPK basal activity in all cell lines, whereas only in H292 ERK was clearly activated by insulin (Fig. 3D and E).

Taken together, insulin enhanced MAPK signaling and proliferation in H292 cells which also had the highest IR expression. In the other two lines, IR expression was lower and insulin effects were smaller or absent.
Down-regulation of IRs by viral transduction

In order to study the role of the IR in the NSCLC cells and its potential contribution to tumor progression in more detail, the expression of this receptor was knocked-down by shRNA. The latter was brought permanently into the cells by lentiviral transduction. In order to confirm reliability of the results, two different shRNAs (sh-IRa, sh-IRb) were employed for IR KD; an unspecific shRNA served as negative control (see Materials and methods).

In four independent experiments, LV-sh-IRa-mediated KD showed prominent reduction of IR expression on D4. Western blotting revealed a KD on protein level of 92 ± 3% in H292, 99 ± 0% in H226 and 99 ± 0% in H460 cells (Fig. 4A and B). Opposed to the strong LV-sh-IRa-caused IR KD, LV-sh-IRb-treated cells revealed a rather low IR KD of about 50–70% (Fig. 4C and D).

Transduction with the IGF1R targeting shRNA led to a down-regulation of IGF1R at the level of mRNA (Fig. 4E) and at protein level (Fig. 4F) in all cell lines (by 86 ± 7% in H292, 67 ± 2% in H226 and 49 ± 2% in H460 cells, Fig. 4G).

Akt and ERK/MAPK signaling in IR KD cells

The cells transduced with shRNA for reduction of IR expression allowed working out via which receptor (IR or IGF1R) insulin activates the signaling pathways as previously described. Akt phosphorylation after KD was determined in all cell lines; MAPK was only studied in H292 cells since the other two lines had revealed no insulin-dependent MAPK activation (see previous discussion). Activation of the signaling pathways was identified by phosphoprotein-western blot analysis of pAkt and pMAPK, respectively (Fig. 5A, B and C).

IR/IGF1R phosphorylation after insulin stimulation was weaker in H292 LV-sh-IRa infected cells than in LV-sh-control cells (Fig. 5A, upper panels), in accordance with the strongly reduced IR expression (Fig. 5A, middle panels). However, the nearly complete loss of IR expression would suggest a much stronger reduction in pIR/pIGF1R. Thus, the observed signal is probably mainly due to pIGF1R since IGF1R expression was not affected (Fig. 5A, lower panels). This assumption is supported by the persistence of a rather strong phosphorylation signal after stimulation with 100 nM insulin. The latter concentration is in the range of the dissociation constant of insulin (around 200 nM; Kurtzhals et al. 2000) for IGF1 receptors. Notably, in H292 already 10 nM insulin caused a clearly detectable receptor phosphorylation in the virtual absence of IR. This effect was only detectable in H292, which is probably due to the fact that these cells have the highest level of IGF1R expression of the cell lines tested (see Fig. 1). Alternatively, other IR-related receptors could play a role.

Although IR KD resulted in a reduction of insulin-induced Akt phosphorylation in all cell lines, pAkt was still...
Figure 3
Insulin is involved in mitogenic signaling in cancer cells. pIGF1R/pIR proteins (A) were studied by western blot analysis. Exposure times were lower for the loading control α-tubulin (1 s) than for pIR/pIGF1R (1 min) to avoid overexposure of the former. The densitometric evaluation was corrected for these different exposure times. Marked signals of activated IR/IGF1R were detected with 10 nM insulin onwards (upper panels). No significant increase in receptor activation was observed with concentrations in picomolar ranges (data not shown). Densitometric analysis of four independent experiments is presented in (B). Representative western blot analysis revealed expression of phospho-Akt and total Akt (C), phospho-p44/42 and total ERK (D). The bar chart in (E) shows quantification of ERK/MAPK phosphorylation. Results presented in bar charts are expressed as percentage of untreated controls, means ± S.E.M. of mostly n=6 independent experiments, measured each in duplicates. Significance of differences is labelled as follows: **P<0.01, ***P<0.001, ###P<0.001 for the comparison groups indicated.
detectable (Fig. 5B). This is probably also due to insulin-induced activation of IGF1R (or related receptors) in case of IR KD.

Insulin-dependent ERK/MAPK signaling pathway after IR KD was only investigated in H292 cells since no insulin-sensitive activation of this protein kinase was detected in the two other cell lines (Fig. 3D and E). In IR KD samples, both basal MAPK activity and its insulin-dependent stimulation were markedly less pronounced than in controls (Fig. 5C), especially stimulatory effects of insulin were significantly reduced, particularly by 67% with 10 nM and 64% with 100 nM insulin (Fig. 5D). This indicates that a large part of the mitogenic ERK/MAPK signaling is mediated via IR and not only via IGF1R.

**Caspase activity in IR KD cells**

Prominent cell death became obvious at D4 and D5 after viral transduction. Cell counts revealed a steady decline in cell number after sh-IRa treatment, down to 2% in H292, 29% in H226 and 7% in H460 of control level at D5 (Fig. 6A). In order to confirm that the reduced cell number is a consequence of IR KD (and not an unspecific effect of this particular shRNA or of lentiviral infection), cells infected with LV-sh-IRb, leading to a lower degree of IR KD, and with LV-sh-IGF1R, leading to IGF1R KD, served as controls.

In four independent experiments cell count remained stable after IGF1R KD in all cell lines tested (data not shown). Partial IR KD also caused a time-dependent decrease in H292 and H226 cell survival, albeit to a lesser extent (D5: 31% and 46%, respectively). In H460 cells, no major difference in cell number after transduction with LV-sh-IRa and LV-sh-IRb at mRNA level (quantitative PCR, four experiments). A representative western blot in H292 comparing IRa and IRb on protein level is shown in (D). Knockdown of IGF1R at mRNA level following transfection with LV-sh-IGF1R was determined by qPCR (E). A representative western blot for IGF1R is shown in (F). The results of densitometric quantification (mean + s.e.m. of four experiments) are provided in (G). The level of statistical significance is indicated as follows: *P<0.05, **P<0.01 and ***P<0.001.
recognized motif does not exclude toxic effects of a so far unrecognized motif. Since potential cytotoxic effects of LV-sh-IRa should not be restricted to a certain cell type, we used primary human pre-adipocytes as a control. The latter are unrelated to bronchial carcinoma cells but are known to be responsive to insulin via IR. The pre-adipocytes were transfected with sh-IRa using the same LV as for the bronchial carcinoma lines. For comparison, LV-sh-control and an shRNA not containing the UGGC motif and targeting an unrelated gene (DPP4) were included. Gene expression and cell count were determined at Days 3 and 5 after infection. At D3, cell count was similar with each shRNA (sh-control, sh-IRa and sh-DPP4, Fig. 6C). At D5, cell count with the specific shRNAs (sh-IRa and sh-DPP4) was around 70% of sh-control. No relevant differences between sh-IRa and sh-DPP4 became evident in all cell lines tested, insulin-induced phosphorylation was diminished in KD cells. KD of insulin receptors was effective (middle panel), whereas IGF1R was present to an equal extent compared to LV-sh-control samples (lower panel).

Microarray-based gene expression profiling and correlation to apoptosis

In order to elaborate the mechanism which could underlie apoptosis induction by the (almost complete) loss of IR, we employed genome-wide expression profiling using an Agilent Whole Genome array (see the ‘Materials and methods’ section for details). The IR KD did not lead to a compensatory upregulation of insulin, IGF1 or IGF1R. Nevertheless, elevated levels of several insulin- and IGF-family members, like insulin-induced genes (INSIG1–2), insulin-like factors (INSL3–5) or IGF-like factors (IGF1L1–3) became evident in all lines tested (Fig. 7A and B). The expression of IGF1L1, known to be enhanced by TNF (Lobito et al. 2011), was up-regulated by about 14 times in H292 IR KD cells. However, the IGF1L1 receptor (IGF1L1R) was down-regulated in the three cell lines. An appreciable increase in IGF2 and IGF2R gene expression of around
100% each was detected in H460 cells. IGF-binding proteins (IGFBP), known to regulate the access of IGF1 and IGF2 to their receptors (Baxter 2014), were markedly enhanced in all samples tested. Furthermore, gene profiling revealed significant changes in the expression of cytokines (Fig. 7C and D). In particular, members of IL1-, CXCL- and CCL-families, as well as IL24 and TNF, were among the most strongly induced genes. In contrast, the interferon family of cytokines was not induced by IR KD.

Apoptosis-inducing cytokines in NSCLC

The observed induction of several cytokines after IR KD (see previous discussions) may constitute a link between IR KD and apoptosis since it is known that certain cytokines can induce or prevent programmed cell death in susceptible cells (Donnelly et al. 2004, Dash et al. 2014). Therefore we tested whether the cytokine up-regulated after IR KD are able to activate caspases in the NSCLC cells studied. Thus, caspase 3/7 assays were performed after cell treatment with those cytokines that were most strongly induced in the DNA microarray experiments and known to be associated with apoptosis: IL6, IL20, IL24 and TNF (Fig. 7E). A significant activation of caspases was elicited by TNF (H292: 237 ± 5%, H226: 130 ± 5% and H460: 231 ± 22%). IL6 and IL24 activated caspases 3/7 in H292 and H226 cells; in H292 cells also IL20 caused an elevated caspase-activity (140%).

LV-sh-IRa caused apoptosis in NSCLC cells. Effects of IR KD on cell survival by count of cell number are shown in (A). Data are expressed as cells/ml. Caspase assay at D3 (B) revealed induction of effector caspases 3 and 7 in IR KD cells compared to negative controls (untreated and UV-sh-control vector-treated cells) and positive control (gemcitabine-treated cells). In order to exclude unspecific effects of LV-sh-IRa on cell survival, we tested the effect of this shRNA on human pre-adipocytes; these cells are an important physiological target of insulin action. Cell count (C) and gene expression (D) were studied in the pre-adipocytes at Day 3 and Day 5 after infection with LV-sh-IRa. LV-sh-control and an shRNA targeting an IR-independent gene, DPP4, were included as comparators. Note that the effect of LV-sh-IRa on cell count in pre-adipocytes was much smaller than in the bronchial carcinoma cells. Furthermore, no relevant induction of cytokines was observed with LV-sh-IRa in pre-adipocytes. The results, presented as percentage of LV-sh-controls, are means ± S.E.M. of three or four independent experiments. *P!0.05, **P!0.01 and ***P!0.001 LV-sh-control vs LV-sh-IRa cells, ##P!0.01, ###P!0.001 LV-sh-control vs LV-sh-IRb cells.
Discussion

In the present study, we have investigated the role of insulin and the IR in three independent human NSCLC lines.

The expression levels of IR were markedly different in the three lines, with H292 expressing the highest level and H460 the lowest. The IR expression level clearly correlated with the magnitude of insulin-induced proliferation. Also, insulin signaling was in good agreement with the respective receptor expression level. The strongest insulin-induced autophosphorylation of IR/IGF1R was observed in H292, followed by H226. In H460, expressing only small amounts of IR, a marked IR/IGF1R autophosphorylation was only observed with the high insulin concentration of 100 nM. This probably means that in H460 cells the observed signaling was mediated by IGF1R, which has a lower affinity to insulin. Regarding more distal signaling, a clear insulin-dependent activation of ERK phosphorylation was only observed in H292 cells, the line with the highest IR expression. For Akt phosphorylation, no clear correlation with IR expression level was observed. In the other two cell lines proliferation rate and MAPK activity were already high under basal conditions so that insulin was obviously unable to further enhance mitogenic signaling.

The phenomenon of basal MAPK activity was described earlier as pathway dysregulation (Fresno Vara et al. 2004, De Luca et al. 2012) and explained by mutations or/and activation via other growth factors, e.g. EGF (Fresno Vara et al. 2004, Rhodes et al. 2008). In conclusion, insulin can increase proliferation at least in some lung cancer cells. Enhancement of tumor cell proliferation was already described for several other tumor types (see Introduction). It was reported that the IGF1 axis is important for proliferation of certain tumor types (reviewed e.g. by Pollak 2008) and Durzyńska et al. (2014)), but it is not known whether insulin effects on tumor progression are mediated via the insulin or the IGF1 receptor. In order to distinguish between insulin actions via IR vs IGF1R we aimed to reduce expression of the IR by lentiviral shRNA KD. Under this condition, insulin-induced Akt signaling was still present but markedly weaker, indicating that this pathway is activated by IR to a large extent and only to a smaller fraction via IGF1R. The same is true for MAPK activation in H292 cells (in the other two cell lines MAPK was not further activated by insulin). These findings indicate that IR plays an important role in these tumor cells and that insulin action in tumors is not mainly mediated by IGF1R.

More importantly, KD of IR caused a dramatic reduction in survival of the tumor cells. This was observed in all three cell lines tested and could therefore not be explained by insulin’s effect on proliferation. Instead, caspase assays revealed that the cells underwent rapid
apoptosis upon KD of the IR. For elucidation of the underlying mechanistic link between IR and apoptosis we studied changes in the gene expression profile following IR KD. It turned out that IR KD suppressed certain cytokines (e.g. TNF and IL24). These cytokines were previously reported to promote apoptosis in certain cancer cells (Donnelly et al. 2004, Dash et al. 2014), and we could show that they also trigger apoptosis in the NSCLC cells studied here. Hence, one can conclude that vice versa presence of IR suppresses the expression of cytokines which can induce apoptosis. Thus, the IR obviously enables tumor cell survival by helping the cells escaping programmed cell death. This mode of IR action has not been previously described.

In contrast to IR KD, KD of IGF1R did not affect lung cancer cell survival. Effects of this kind might explain why cancer treatment directed against the IGF1R has not yet affected cancer cell survival. Effects of this kind might explain why cancer treatment directed against the IGF1R has not yet revealed the expected success (Vincent et al. 2013). Our data show that not only PI3K/Akt kinases activity (Kim et al. 2001), but also IR functionality directly prevents NSCLC cells from apoptosis by presenting a mechanistic link. This implies that the IR could be highly important for the survival of malignant cells.

The question remains why IR KD affects cell survival even in the apparent absence of an activating ligand (i.e. insulin). It was reported that activation of Akt/PKB signaling is associated with the inhibition of apoptosis (reviewed by Galetic et al. (1999)). However, our findings make involvement of Akt signaling unlikely because IR KD did not alter basal Akt activity (measured as pAkt). Alternatively, it is conceivable that IR KD liberates signaling proteins which in turn can be used by other growth factor receptors and thereby enhance their signaling. Future studies will investigate this link in more detail.

In the context of a diabetes mellitus therapy with inhalable insulin, safety concerns about tumor promotion (Siekmeier & Scheuch 2008) are further supported. Our results imply that the anabolic hormone insulin has the potential of promoting the growth of pre-existing neoplasms.

Exposure to supraphysiological insulin concentrations in the oropharynx and lungs (Santos Cavaiola & Edelman 2014), which probably lasts over decades, consequently bear a risk to support malignancies. Especially patient groups with an increased prevalence of cancer entities in the respiratory system, e.g. smokers, ex-smokers, people being exposed to air pollution or patients with chronic inflammatory diseases, could be affected. No clinical trials of sufficient duration and carried out in a sufficiently large sample size are available to date to address this open question.

Our data further reveal that in NSCLC cells, the IR has a crucial role in cancer cell survival. We therefore conclude that the IR represents an interesting therapeutic target in the treatment of neoplasia.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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