HEPARIN AFFIN REGULATORY PEPTIDE/PLEIOTROPHIN MEDIATES FIBROBLAST GROWTH FACTOR 2 STIMULATORY EFFECTS ON HUMAN PROSTATE CANCER CELLS

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Running title: FGF2 acts on human prostate cancer cells through HARP
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Fibroblast growth factor 2 (FGF2) is a pleiotropic growth factor that has been implicated in prostate cancer formation and progression. In the present study we found that exogenous FGF2 significantly increased human prostate cancer LNCaP cell proliferation and migration. Heparin affin regulatory peptide (HARP) or pleiotrophin seems to be an important mediator of FGF2 stimulatory effects, since the latter had no effect on stably transfected LNCaP cells that did not express HARP. Moreover, FGF2, through FGFR, significantly induced HARP expression and secretion by LNCaP cells and increased luciferase activity of a reporter gene vector carrying the full length promoter of HARP gene. Using a combination of Western blot analyses, as well as genetic and pharmacological inhibitors, we found that activation of FGFR by FGF2 in LNCaP cells leads to NAD(P)H oxidase-dependent hydrogen peroxide production, phosphorylation of ERK1/2 and p38, activation of AP-1, increased expression and secretion of HARP and finally, increased cell proliferation and migration. These results establish the role and the mode of activity of FGF2 in LNCaP cells and support an interventional role of HARP in FGF2 effects, providing new insights on the interplay among growth factor pathways within prostate cancer cells.

Human prostate relies on a range of growth factors for its normal growth and development. It has been hypothesized that the progression to malignant growth of the prostate is associated with a deregulated expression of various growth factors and/or their receptors. In the last few years, signaling pathways of the fibroblast growth factor (FGF) family have been subject to intense investigation, since there is increasing evidence that alterations of FGFs and/or FGF receptors (FGFRs) may play an important role in initiation and progression of prostate cancer (reviewed in 1).

FGF2 is a broad spectrum and pleiotropic mitogen for growth and differentiation, affecting epithelial and endothelial cells, smooth muscle cells and osteoblasts (2). FGF2 incites tumor angiogenesis (2, 3), is expressed by various tumor cell lines (4-6) and seems to be biologically important in tumor progression and metastasis (7). Clinically, FGF2 is implicated in malignant growth of the prostate (8) and elevated levels of serum FGF2 are detected in prostate cancer patients (9). Nevertheless, inhibition of FGF2 expression has been associated with increased levels of vascular endothelial growth factor (VEGF) in poorly differentiated TRAMP prostate tumors, implying that other factors can at least partly overcome loss of FGF2 stimulation (7).

Heparin affin regulatory peptide (HARP), also known as pleiotrophin, exhibits important biological activities, being a mitogenic, anti-apoptotic, transforming, chemotactic and angiogenic factor (10-13). A potential role of HARP in human cancers was suggested after the detection of HARP mRNA and/or protein in various human cancer cell lines and tumor specimens of diverse origin (reviewed in 11). We have recently identified HARP as an important autocrine growth factor for the LNCaP
prostate cancer cell line, and as a paracrine growth factor implicated in prostate cancer cell-induced angiogenesis in vivo and in vitro (12).

The goal of this study was to evaluate the effect of FGF2 on human prostate cancer LNCaP cell proliferation and migration and investigate whether HARP is implicated in FGF2-mediated activation of LNCaP cells. Our data demonstrate an FGF2-induced H2O2-dependent HARP up-regulation, resulting in increased LNCaP cell proliferation and migration and suggest that H2O2 generation in response to FGFR stimulation originates from NAD(P)H oxidase activation and results in AP-1-mediated HARP expression.

Experimental Procedures

Materials - The FGFR specific inhibitor SU-5402, the antioxidant sodium pyruvate and the NAD(P)H oxidase specific inhibitor 4'-hydroxy-3'-methoxyacetophenone (apocynin) were purchased from Calbiochem, Biochrom AG and Fluka, respectively. The H2O2 scavenger catalase, the NAD(P)H oxidase inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and the xanthine oxidase inhibitor allopurinol, were purchased from Sigma. The MEK inhibitor U0126 and the selective p38 inhibitor SB202190 were purchased from Calbiochem, Biochrom AG and Biosource, respectively. All the inhibitors and antioxidants at the concentrations used were not toxic to the cells. Recombinant human FGF2 and HARP were prepared in Escherichia coli and purified to homogeneity by sequential heparin affinity and Mono S chromatographies, as previously described (12, 14).

Cell culture - The human prostate cancer epithelial cell line LNCaP (ATCC) was grown routinely in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 2.5 µg/ml amphotericin B. Cultures were maintained at 37°C, 5% CO2 and 100% humidity. For treatment with FGF2, LNCaP cells were grown in RPMI-1640 supplemented with 2% FBS. Other treatments were initiated 30 min prior to FGF2 treatment. Development of stably transfected LNCaP cell lines, PC-LNCaP bearing the appropriate control vector (pCDNA3.1) and AS-LNCaP expressing antisense HARP has been previously described (12).

Transient Transfection and Luciferase Assay - LNCaP cells were transfected with wild-type hHARPpro2.3-Luc reporter plasmid containing about 2.3 kb of the human HARP 5'-flanking region or constructs mutated at either one or both activator protein-1 (AP-1) binding sites, as previously described (15). After removing the transfection medium, FBS was added to a final concentration of 2% and 24 h later the culture medium was replaced with fresh medium containing FGF2 (10 ng/ml). Cells were harvested and luciferase activity was determined using the Luciferase Reporter Gene Assay (Roche, Germany). Cell lysates were analyzed for protein content using the Bradford method, and luminescence units were normalized for total protein content.

Decoy oligonucleotide (ODN) technique - Double-stranded ODNs were prepared from complementary single-stranded phosphorothioate-bonded ODNs (Biopaths, Greece). The efficiency of the hybridization reaction was verified (over 95%) and ODN uptake was achieved using jetPEI™, as previously described (15). The maximally effective concentration of ODNs was determined to be 450 nM and the optimal incubation time with the cells was 4 h.

Assay of intracellular reactive oxygen species (ROS) production - ROS were assayed using the ROS-sensitive fluorescent dye, 5(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, Fluka). LNCaP cells were seeded in 6-well plates and the second day after seeding were grown in serum-free RPMI-1640 containing different concentrations of FGF2 for different periods of time. Treatments of LNCaP cells with antioxidants were initiated 30 min prior to FGF2 stimulation. Plates were washed with PBS, and then incubated in the dark for 15 min in Ham’s F-12 lacking phenol red containing 50 µM carboxy-H2DCFDA. As a positive control, cells were loaded with carboxy-H2DCFDA for 15 min as above, the medium was discarded, and H2O2 (5 µM) was added for different periods of time. At the end of the incubation period, intracellular ROS generation was quantified spectrophotometrically by measurement of carboxy-dichlorofluorescein (DCF), the fluorescent oxidation product of carboxy-H2DCFDA, in soluble extracts of cells as previously described (16). In brief, after treatment as described above, the cells were released with trypsin/EDTA and lysed. Soluble extracts were prepared by centrifugation for removal of cell debris and fluorescence intensity was determined spectrophotometrically, using an
excitation wavelength of 485 nm and emission wavelength of 500 nm. Cell lysates were analyzed for protein content using the Bradford method, and carboxy-DCF fluorescence was normalized for total protein content.

**Cell proliferation assay** - In order to measure the number of cells, the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-dimethyltetrazolium bromide (MTT) assay was used, as previously described (12). LNCaP cells were seeded at 5x10^3 cells/well in 24-well tissue culture plates, in RPMI-1640 supplemented with 10% FBS. Twenty-four hours later, cells were starved in medium supplemented with 2% FBS for 16 h. Culture medium was replaced with fresh medium containing substances tested and the number of cells was determined. Results were always confirmed by direct counting of cells under the microscope, using a standard haemocytometer.

**Migration assay** - Migration assays were performed as previously described (12) in 24-well microchemotaxis chambers (Costar, Avon, France) using uncoated polycarbonate membranes with 8 µm pores. Briefly, LNCaP cells were harvested and resuspended at 10^4 cells/0.1 ml in RPMI-1640 supplemented with 0.5% BSA. The bottom chamber of each transwell unit contained 0.6 ml of RPMI-1640 supplemented with 0.5% BSA and 0.5% FBS. The plates were incubated for 20 h at 37°C and the filters were fixed with saline-buffered formalin and stained with 0.33% toluidine blue solution. The cells that migrated through the filter were quantified by counting the entire area of each filter, using a grid and an Optech microscope at a 20x magnification.

**Evaluation of DNA binding activity of AP-1 by enzyme-linked immunosorbent assay (ELISA)** - The DNA binding activity of AP-1 was quantified by ELISA using the Trans-AM AP-1 Transcription Factor Family Assay kit (Active Motif Europe, Belgium), as previously described (15). Briefly, nuclear extracts were prepared using Nuclear Extract Kit (Active Motif Europe) and incubated in 96-well plates coated with immobilized oligonucleotide, containing a consensus binding site for AP-1 (wild-type TPA Response Element, TRE). AP-1 binding to the target oligonucleotide was detected by incubation with primary antibodies specific for c-Fos, Fos-B, Fra-1, Fra-2, JunB, JunD or the activated form of c-Jun, visualized with anti-IgG horseradish peroxidase conjugate and developing solution, and quantified at 450 nm with a reference wavelength of 655 nm. For the competition analysis, nuclear extracts were pre-incubated with the AP-1 like motifs of the HARP promoter and the respective mutated sequences or the wild type TRE.

**Western blot analysis** - The presence of HARP in the cell culture medium was investigated as previously described (12). Briefly, the conditioned medium of the cells was incubated overnight with 100 µl of heparin-Sepharose (Amersham Pharmacia Biotech, UK) at 4°C with continuous agitation. Bound proteins were eluted with 50 µl of Laemmli sample buffer under reducing conditions, fractionated on 17.5% SDS-PAGE and transferred to Immobilon P membranes. Blocking was performed by incubating the PVDF membranes with 3% BSA in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T). The membranes were then incubated with 45 ng/ml affinity purified anti-HARP antibody in TBS-T for 1 h at room temperature under continuous agitation and then with horseradish peroxidase conjugated rabbit anti-goat IgG (Sigma, Greece) at a dilution of 1:7,500 in TBS-T, for 1 h at room temperature under continuous agitation. In order to study activation of extracellular signal-regulated kinases (ERK)1/2 and p38, cell lysates were analyzed by SDS-PAGE and transferred to Immobilon P membranes. Blocking was performed by incubating the membranes with 5% non-fat dry milk in Tris buffered saline (TBS) pH 7.4 containing 0.1% Tween-20 (TBS-T), for 2 h at room temperature. Membranes were further incubated in primary antibodies for 18 h at 4°C under continuous agitation, as following: anti-phosphoERK1/2 antibody (phospho-44/42 mitogen activated protein kinase, MAPK, on Thr202/Tyr204, Cell Signaling, U.S.A.) or anti-phosphop38 (phospho-p38 MAP Kinase on Thr180/Tyr182, Cell Signaling) at 1:1,000 dilution in TBS-T containing 5% BSA, anti-ERK1/2 antibody (Anti-MAP Kinase ½, Upstate, U.S.A.) at 1:5,000 dilution in TBS-T containing 3% non-fat dry milk and anti-p38 antibody (p38 MAP Kinase antibody, Cell Signaling) at 1:1,000 dilution in TBS-T containing 5% BSA. Membranes for ERK 1/2 were further incubated with horseradish peroxidase conjugated anti-rabbit IgG (Sigma) at a dilution of 1:12,500 in TBS-T, for 1 h at room temperature under continuous agitation. Membranes for p38 were further incubated with horseradish peroxidase conjugated anti-rabbit IgG (Cell Signaling) at a dilution of 1:2,000 in TBS-T containing 5% non-
fat dry milk. Detection of immunoreactive bands was performed by the ChemiLucent Detection System Kit (Chemicon International Inc., CA), according to the manufacturer’s instructions. Blots for phospho-ERK1/2 and phospho-p38 were stripped and subjected to subsequent Western blotting for ERK1/2 and p38 respectively. The protein levels that corresponded to HARP were quantified using the ImagePC image analysis software (Scion Corporation, Frederick, MD).

**Results**

**HARP expression is required for FGF2-induced LNCaP cell proliferation and migration** - We initially studied the effect of FGF2 on the proliferation and migration of human prostate cancer LNCaP cells. As shown in Fig. 1A, FGF2 significantly increased the number of LNCaP cells in a concentration-dependent manner, 48 h after its addition into the LNCaP cell culture medium, with the maximum effect observed at 10 ng/ml (Fig. 1A). Similarly, FGF2 significantly induced LNCaP cell migration (Fig. 1D). Treatment of LNCaP cells with the FGFR tyrosine kinase activity inhibitor SU-5402 (17) for 30 min prior to stimulation with FGF2, resulted in marked attenuation of FGF2 mitogenic effect (Fig. 1B).

We have recently shown that endogenous HARP expression plays a key role in LNCaP cell proliferation and migration (12). To investigate the implication of HARP on FGF2-induced LNCaP cell proliferation and migration, we used LNCaP cells expressing either antisense HARP (AS-LNCaP) or the appropriate control vector (PC-LNCaP). FGF2 significantly increased PC-LNCaP cell proliferation and migration, similarly to its effect on non-transfected LNCaP cells, while it had no effect on AS-LNCaP cells (Fig. 1C and D). On the other hand, VEGF exerted similar mitogenic effect on both PC- and AS-LNCaP cells (Fig. 1C), showing that the lack of expression of HARP interrupts FGF2 proliferative effect in a selective manner. The mitogenic effect of exogenously added VEGF in LNCaP cells is in agreement with previous reports (18).

Exogenous addition of human recombinant HARP stimulated LNCaP cell proliferation to a similar degree with FGF2 (12 and Fig.1E). Simultaneous addition of both factors, at concentrations that have the maximum effect (10 ng/ml for each factor), did not cause any further increase in LNCaP cell proliferation compared with the effect of each factor alone (Fig. 1E).

**FGF2 induces the expression and secretion of HARP by LNCaP cells, through transcriptional activation of the corresponding gene** - In order to study whether FGF2 affects HARP secretion by LNCaP cells, we treated the cells with FGF2 (10 ng/ml) and measured HARP protein amounts at different time points after the addition of FGF2 into the cell culture medium. HARP protein levels secreted into the culture medium of LNCaP cells were significantly increased 24 and 48 h after the addition of FGF2 (Fig. 2A). Similarly, HARP mRNA levels were also increased 24 and 48 h after treatment of LNCaP cells with FGF2, as revealed by semi-quantitative RT-PCR (data not shown). In order to evaluate whether the effect of FGF2 on HARP is also observed at the transcriptional level, we used a plasmid construct containing the full length promoter of the human HARP gene fused to a luciferase reporter gene, to transfect LNCaP cells as described in Methods. Reporter activity was increased in response to FGF2 in LNCaP cells since the first 2 h after its addition into the cell culture medium, and was maximal at 6 h (Fig. 2C). Disruption of FGFR signaling with SU-5402 abolished FGF2-induced HARP protein secretion (Fig. 2B) and transcriptional activation of HARP gene (Fig. 2D).

**AP-1 mediates FGF2-induced transcriptional activation of HARP gene** - We have recently demonstrated the involvement of AP-1 in the regulation of HARP gene expression in LNCaP cells (15). We therefore examined a possible implication of AP-1 in the FGF2-induced HARP up-regulation. Transfection of LNCaP cells with AP-1 ODNs prior to stimulation with FGF2, resulted in marked attenuation of FGF2-induced protein up-regulation. On the contrary, ODNs containing mutated AP-1 consensus sequence had no effect (Fig. 3A). We next assessed the contribution of the two AP-1-like motifs of the HARP promoter to the increased transcription of HARP gene in FGF2-stimulated LNCaP cells.
We used constructs with point mutations in either one (AP-1-mt-I and AP-1-mt-II) or both (AP-1-dmt) AP-1-like motifs. Our analysis revealed that FGF2 had no effect on the reporter activity of these constructs (Fig. 3B), suggesting a pivotal role of these two motifs in FGF2-stimulated transcription of HARP gene in LNCaP cells.

To evaluate the modification of DNA binding activity of AP-1 in response to FGF2, we performed an AP-1-subunit-specific ELISA analysis. Nuclear extracts derived from unstimulated and FGF2-stimulated LNCaP cells were used. As shown in Fig. 3C, Fra-1, c-Fos, JunD and phospho-c-Jun DNA binding activity were up-regulated 5 h post stimulation of LNCaP cells with FGF2 (10 ng/ml). In order to figure out the AP-1 subunits that form the FGF2-induced active complex on HARP promoter, a competitive ELISA was carried out. Nuclear extracts were pre-incubated with the AP-1 like motifs of the HARP promoter and the respective mutated sequences or the wild type TRE. AP-1 binding motifs of the HARP promoter significantly decreased binding of Fra-1, phospho-c-Jun and JunD, while the binding of c-Fos, FosB, Fra-2 and JunB was not affected. Mutated AP-1 binding like motifs of the HARP promoter had no effect (Fig. 3C).

FGF2 stimulates NAD(P)H oxidase-dependent production of H2O2 in LNCaP cells - Since the effect of FGF2 on the activation of HARP gene promoter resembles that of exogenously added H2O2 (15) and taking into consideration that emerging evidence points to the involvement of ROS as signaling intermediates for receptor tyrosine kinase-linked growth factors (19, 20), we tested whether FGF2 induces H2O2 production in LNCaP cells. Exposure of LNCaP cells to FGF2 for different periods of time resulted in a rapid concentration-dependent increase in carboxy-DCF fluorescence, which was maximal 5 min after stimulation and returned to baseline values after 30 min (Fig. 4A and B). SU-5402 prevented FGF2-induced carboxy-DCFH oxidation, indicating the involvement of FGFR activation (Fig. 4C). Pretreatment of LNCaP cells with sodium pyruvate or catalase inhibited the FGF2-induced increase in carboxy-DCF fluorescence (Fig. 4D), indicating the involvement of H2O2 in carboxy-DCFH oxidation. To evaluate the enzymatic origin of H2O2 generation in response to FGF2, we used apocynin and AEBSF, two potent inhibitors of NAD(P)H oxidase. Treatment of LNCaP cells with apocynin or AEBSF prior to stimulation with FGF2, completely abolished the FGF2-induced production of H2O2 (Fig. 4D). In contrast, FGF2 induced DCF-fluorescence was not affected by treatment of cells with allopurinol (1 mM), a xanthine oxidase inhibitor (Fig. 4D). These results indicate a role for NAD(P)H oxidase in FGF2-stimulated H2O2 production in LNCaP cells.

Activation of NAD(P)H oxidase and generation of H2O2 are required for FGF2-induced HARP secretion and LNCaP cell proliferation - We next studied whether FGF2 induced HARP secretion and LNCaP cell proliferation were mediated by elevation of H2O2 levels. As shown in Fig. 5, pretreatment of LNCaP cells with sodium pyruvate or apocynin eliminated both FGF2-induced HARP secretion and LNCaP cell proliferation.

ERK1/2 and p38 transduce the signal triggered by FGF2 in LNCaP cells - In order to evaluate the possible mechanism through which FGF2 elicits its downstream effects, we investigated the involvement of activation of MAPKs in FGF2 induced HARP secretion and LNCaP cell proliferation. Given that FGF2 induces binding of specific AP-1 complexes to the HARP gene promoter leading to HARP up-regulation, we went on to determine the dependence of active AP-1 complexes upon stimulation of MAPKs pathways. We used U0126 (10 µM) and SB202190 (10 µM), inhibitors of ERK1/2 activation and p38 activity respectively. LNCaP cells were treated with the inhibitors for 30 min prior to stimulation with FGF2 and the nuclear extracts were submitted to AP-1-subunit-specific ELISA analysis. As shown in Fig. 6C, blockade of ERK1/2 or p38 significantly attenuated the binding activity of the AP-1 subunits responsible for HARP gene up-regulation in response to FGF2. In the same line, inhibition of either ERK1/2 activation or p38 activity resulted in abrogation of FGF2-stimulated HARP secretion (Fig. 6D).
DISCUSSION

Current knowledge supports the interplay of different growth factors and growth modulators as a usual phenomenon in various cell types (21). We have recently identified HARP as an important autocrine growth factor for human prostate cancer LNCaP cells (12). In the present study, we evaluated a possible implication of HARP in FGF2-induced biologic effects. Initially, we observed a delay in the proliferative response to FGF2, which was observed only after 48 h, and speculated an indirect involvement of other stimuli. Moreover, FGF2 stimulatory actions on human prostate cancer cells were attenuated in LNCaP cells expressing antisense HARP, pointing to the requirement of HARP expression for FGF2-induced LNCaP cell proliferation and migration. Our hypothesis of an interventional role of HARP in FGF2 effects was reinforced by the observation that FGF2 resulted in activation of HARP transcription and increased protein levels in LNCaP (this study) and PC-3 prostate cancer epithelial cell lines (Hatziapostolou et al., unpublished data). The observation that either each growth factor alone or concomitant addition of the two factors resulted in a similar induction of cell proliferation, further supports the notion that HARP mediates the stimulatory effects of FGF2 on LNCaP cells. A correlated expression of FGF2 and HARP has been previously suggested given their simultaneously elevated levels in the serum of testicular cancer patients (22) and a correlated expression in gliomas (23). It is the first time, however, that a direct interplay between the two growth factors is being evidenced.

Although FGF2 drives multiple intracellular signaling pathways that are involved in cell proliferation and migration, its ability to regulate these processes depends on the type of FGFR that triggers a specific signal transduction pathway. All four types of FGFRs are expressed in prostate cancer epithelial cells at variable frequencies (1). Among them, FGFR1 and FGFR4 have been linked to prostate cancer progression. In fact, increased expression or activation of FGFR1 has been correlated with transformation of prostate cancer cells (8, 24) and tumor progression (25) and blockade of FGFR1 tyrosine kinase activity by SU-5402 resulted in a decrease in the growth of LNCaP tumors in nude mice (26). In the present study we showed that SU-5402 impaired FGF2-stimulated HARP secretion and gene transcriptional activation, as well as LNCaP cell proliferation, suggesting that binding of FGF2 to FGFR1 drives a downstream signal responsible for HARP up-regulation and subsequent LNCaP cell proliferation.

We have recently demonstrated the involvement of two AP-1 response elements in the transcriptional regulation of the human HARP gene (15). In order to investigate a possible implication of AP-1 in HARP protein up-regulation by exogenously added FGF2, AP-1 activity inhibited by decoy oligonucleotides resulted in marked decrease of FGF2-induced HARP secretion. Both AP-1-like binding sites were required for the transcriptional up-regulation of HARP in FGF2-treated cells and the active AP-1 complex that binds to the HARP promoter in response to FGF2 consists of Fra-1, JunD and phospho-c-jun, similar to the effect of exogenously added H2O2 on LNCaP cells (15). Therefore, we examined whether the effects of FGF2 on HARP expression and LNCaP cell functions are mediated by ROS. Indeed, FGF2 induced intracellular ROS generation in LNCaP cells through activation of FGFR. Several previous studies show the generation of ROS in a variety of cells upon stimulation by different growth factors (19, 27), however, this has not been previously reported for FGF2-induced activation of FGFR. The ROS produced after activation of FGFR seems to be H2O2 since the effect was completely inhibited by catalase and sodium pyruvate, which have been previously shown to reduce cellular H2O2 concentrations (28, 29). Moreover, FGF2-stimulated increase in intracellular H2O2 concentration was completely blocked by NAD(P)H oxidase (but not xanthine oxidase) inhibitors, suggesting that superoxide is probably the initial ROS produced by FGF2, rapidly dismutating to H2O2.

Earlier studies suggest that ROS generation through the novel NAD(P)H oxidases, called Nox, may be a general property of prostate cancer cells and support a causal role of Nox-derived ROS in cell growth (30-32). More specifically, LNCaP prostate cancer cells express components of NAD(P)H oxidases (Nox1 and 5) and LNCaP cell growth is inhibited by antioxidant strategies (30). Furthermore, a series of cell lines developed from LNCaP prostate cancer cells that demonstrate increasing tumor and metastatic potential show increased Nox 1 and a parallel increase in H2O2 levels (32).
In the present study, exposure of LNCaP cells to H$_2$O$_2$ scavengers or NAD(P)H oxidase inhibitors resulted in abrogation of FGF2-induced HARP secretion and consequent cell proliferation, suggesting that NAD(P)H oxidase activity and H$_2$O$_2$ are essential components of the signaling cascade triggered by FGF2.

Mounting evidence has pointed to the involvement of ROS as intracellular second messengers in tyrosine kinase receptor signaling, which regulate activation of various MAPKs (33, 34). Upon activation, MAPKs can phosphorylate and activate their specific molecular targets, such as transcription factors that can then regulate the expression of specific genes and transport the signals responsible for increased proliferative and/or migratory response of different cell types (16, 35, 36). We found that FGF2 caused a rapid and sustained increase in the levels of phosphorylation of both ERK1/2 and p38 in LNCaP cells, in line with previous reports (37-39). Activation was abrogated by apocynin, suggesting that H$_2$O$_2$ generation by NAD(P)H oxidase is necessary for FGF2 stimulatory effect on MAP kinases.

All MAPKs regulate AP-1 in terms of transcriptional activation, protein stability and biochemical activity (40-41). To establish the molecular link between FGF2-activated MAPKs and AP-1/HARP, we used inhibitors of p38 and ERK1/2 pathways and found that blockade of either cascade attenuated the positive effect of FGF2 on the DNA binding activity of AP-1 complexes composed of c-Jun, Fra-1 and JunD and the consequent secretion of HARP by LNCaP cells. Although from the present study we do not have data on a selective effect of each MAPK on specific AP-1 subunits, our results reflect the requirement of a coordinated activation of these pathways for HARP expression.

In conclusion, as illustrated in Fig. 7, we show for the first time that exogenous FGF2 stimulates LNCaP cells to produce HARP and that FGF2 signaling is conveyed via NAD(P)H oxidase-dependent H$_2$O$_2$ generation, phosphorylation of ERK1/2 and p38 MAPKs and activation of the transcription factor AP-1. The present study reflects the many-sidedness of growth factor pathways within prostate cancer and is the first to demonstrate an important relation between FGF2 and HARP in regulating prostate cancer epithelial cell functions. Further in vivo and in vitro studies are in progress to establish the importance of HARP in prostate cancer growth and angiogenicity and the signalling involved in HARP-induced LNCaP cell proliferation and migration.

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FOOTNOTES

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Fig. 1. Effect of FGF2 on LNCaP cell proliferation and migration. Results are expressed as mean ± S.E.M. of the number of cells. FGF2, cells treated with FGF2 (10 ng/ml); HARP, cells treated with HARP (10 ng/ml); SU-5402, cells treated with the specific inhibitor of FGFR tyrosine kinase activity SU-5402 (5 µM); SU-5402+FGF2, LNCaP cells treated with SU-5402 for 30 min before the addition of FGF2; LNCaP, non-transfected LNCaP cells; PC-LNCaP, LNCaP cells transfected with the plasmid containing only the neomycin resistance gene; AS-LNCaP, LNCaP cells transfected with the plasmid containing antisense HARP. Asterisks denote a statistically significant difference from the corresponding untreated cells. *P<0.05, ***P<0.001.

Fig. 2. FGF2 induces the expression and secretion of HARP by LNCaP cells through FGFR activation. A and B, representative pictures of Western blot analyses of LNCaP culture medium for HARP of 4 independent experiments. HARP protein amounts were quantified by densitometric analysis of the corresponding band in each lane. Results are expressed as mean ± S.E.M. of the % change of the amounts of HARP protein in treated compared with the untreated cells. C and D, reporter activities of hHARPpro2.3-Luc in LNCaP cells are expressed as mean ± S.E.M. of relative luciferase units (RLU) per mg of total protein or mean ± S.E.M. of the % change of luciferase relative activity in treated compared with the untreated cells. FGF2, cells treated with FGF2 (10 ng/ml); SU-5402, cells treated with SU-5402 (5 µM); SU-5402+FGF2, LNCaP cells treated with SU-5402 for 30 min before the addition of FGF2. Asterisks in A and C, denote a statistically significant difference from the untreated cells at the corresponding time points. *P<0.05, **P<0.01, ***P<0.001.

Fig. 3. FGF2-induced HARP expression/secretion is mediated through AP-1. A, representative picture of Western blot analysis of LNCaP culture medium for HARP of 4 independent experiments. HARP protein amounts were quantified by densitometric analysis of the corresponding band in each lane. Results are expressed as mean ± S.E.M. of the % change of the amounts of HARP protein in cells treated with AP-1 ODNs (AP-1) compared with the cells treated with the mutated AP-1 ODNs (mutAP-1). B, reporter activities of AP-1-mutated constructs of the HARP promoter in response to FGF2 in LNCaP cells. Reporter activities were evaluated in untreated and FGF2-stimulated LNCaP cells and expressed as mean ± S.E.M. of relative luciferase units (RLU) per mg of total protein. C, DNA binding activities of Jun and Fos family members after stimulation of LNCaP cells with FGF2 (10 ng/ml) in the absence or presence of AP-1-like motifs or the respective mutated sequences (mut AP-1-like motifs) of the human HARP promoter. Results are expressed as mean ± S.E.M. of the % change of the binding of each AP-1 subunit compared with the untreated cells. Asterisks denote a statistically significant difference from the untreated cells. *P<0.05, ***P<0.001.

Fig. 4. FGF2 induces NAD(P)H oxidase-mediated H2O2 generation in LNCaP cells. The ratio carboxy-DCF fluorescence/mg total protein was calculated for each sample. Results are expressed as mean ± S.E.M. of the % change of carboxy-DCF relative fluorescence in FGF2-stimulated cells compared with the untreated cells in A and B and as mean ± S.E.M. of carboxy-DCF fluorescence per mg of total protein in C and D. F, cells treated with FGF2 (10 ng/ml); SU, cells treated with SU-5402 (5 µM); AL, cells treated with allopurinol (1 mM); SP, cells treated with sodium pyruvate (1 mM); CT, cells treated with catalase (500 Units/ml); AP, cells treated with apocynin (0.5 mM); AE, cells treated with AEBSF (200 µM). SU-5402 and the antioxidants used were added to the cells 30 min prior to addition of FGF2. Asterisks in A and B, denote a statistically significant difference from the untreated cells. Asterisks in D, denote a statistically significant difference from the cells treated with FGF2. *P<0.05, **P<0.01, ***P<0.001.

Fig. 5. NAD(P)H oxidase-mediated H2O2 production is required for FGF2-stimulatory effects on LNCaP cells. A and B, representative pictures of Western blot analyses of LNCaP culture medium for HARP of 4 independent experiments. HARP protein amounts were quantified by densitometric analysis of the corresponding band in each lane. Results are expressed as mean ± S.E.M. of the % change of the amounts of HARP protein in treated compared with the untreated cells. C and D, results are expressed as mean ± S.E.M. of the number of cells. FGF2, cells treated with FGF2 (10 ng/ml); SP,
cells treated with sodium pyruvate (1mM); Apocynin, cells treated with apocynin (0.5 mM). LNCaP cells were incubated with the antioxidants for 30 min before the addition of FGF2. Asterisks denote a statistically significant difference from the untreated cells. *P<0.05, ***P<0.001.

Fig. 6. FGF2 exerts its stimulatory effects on LNCaP cells via ERK 1/2 and p38 activation. A and B, representative pictures of Western blot analyses of 2 independent experiments for ERK 1/2 and p38 phosphorylation. C, DNA binding activities of Jun and Fos family members after stimulation of LNCaP cells with FGF2 (10 ng/ml) in the absence or presence of MAPK inhibitors. Results are expressed as mean ± S.E.M. of the % change of the binding of each AP-1 subunit compared with untreated cells. D, representative picture of Western blot analysis of LNCaP culture medium for HARP of 3 independent experiments. HARP protein amounts were quantified by densitometric analysis of the corresponding band in each lane. Results are expressed as mean ± S.E.M. of the % change of the amounts of HARP protein in treated compared with the untreated cells. LNCaP cells were incubated with apocynin or MAPKs inhibitors for 30 min before the addition of FGF2. Asterisks in C and D denote a statistically significant difference from cells treated with FGF2 and untreated cells, respectively. *P<0.05, **P<0.01, ***P<0.001.

Fig. 7. Schematic diagram of the suggested pathway for FGF2 stimulatory effect on LNCaP cell functions. FGF2, which signals through tyrosine kinase receptor (FGFR) activation, increases the levels of NAD(P)H oxidase-derived H₂O₂ provoking the activation of ERK1/2 and p38 MAPKs. ERK1/2 and p38 pathways are functionally involved in AP-1-mediated harp gene transcriptional activation and the consequent HARP protein release. The latter seems to affect LNCaP cell proliferation and migration through an as yet non identified mechanism.
Fig. 3

A

HARP

AP-1

FGF2

mutAP-1

AP-1

mut AP-1 + FGF2

AP-1 + FGF2

B

% Change of control

P < 0.01

P < 0.01

P < 0.01

P < 0.01

FGF2

mutAP-1

AP-1

AP-1 + FGF2

C

% Change of control

P < 0.01

P < 0.01

P < 0.01

AP-1 + FGF2

mutAP-1 + FGF2

FGF2 w/o s.oligos

Control
Fig. 5

**A**

HARP

FGF2 + SP - + + -

% Change of control

Control FGF2 SP SP

P < 0.01

**B**

HARP

FGF2 + Apocynin - + + +

% Change of control

Control FGF2 Apocynin Apocynin

P < 0.01

**C**

Cell number x 10^3

Control FGF2 SP SP

P < 0.001

**D**

Cell number x 10^3

Control FGF2 Apocynin Apocynin

P < 0.001
Heparin affin regulatory peptide/pleiotrophin mediates fibroblast growth factor 2 stimulatory effects on human prostate cancer cells

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