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Bimoclomol, a heat shock protein co-inducer, acts by the prolonged activation of heat shock factor-1☆,☆☆

Judit Hargitai,[a] Hannah Lewis,[b] Imre Boros,[c] Tímea Rácz,[a] András Fiser,[d] István Kurucz,[a,1] Ivor Benjamin,[e] László Vígh,[c] Zoltán Pénzes,[a] Péter Csermely,[a,f,*] and David S. Latchman[b]

[a] *Biorex R&D Co., H-8201 Veszprém, P.O. Box. 348, Hungary*
[b] *Medical Molecular Biology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK*
[c] *Department of Biochemistry, Biological Research Center of Szeged, Hungarian Academy of Sciences, P.O. Box. 521, H-6701 Szeged, Hungary*
[d] *Laboratory of Molecular Biophysics, Pels Family Center for Biochemistry and Structural Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA*
[e] *Department of Internal Medicine, University of Texas, Southwestern Medical Center, Dallas, TX 75235-8573, USA*
[f] *Department of Medical Chemistry, Semmelweis University, P.O. Box. 260, H-1444 Budapest 8, Hungary*

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Abstract

The novel hydroxylamine derivative, bimoclomol, has been shown previously to act as a co-inducer of several heat shock proteins (Hsp-s), enhancing the amount of these proteins produced following a heat shock compared to heat shock alone. Here we show that the co-inducing effect of bimoclomol on Hsp expression is mediated via the prolonged activation of the heat shock transcription factor (HSF-1). Bimoclomol effects are abolished in cells from mice lacking HSF-1. Moreover, bimoclomol binds to HSF-1 and induces a prolonged binding of HSF-1 to the respective DNA elements. Since HSF-1 does not bind to DNA in the absence of stress, the bimoclomol-induced extension of HSF-1/DNA interaction may contribute to the chaperone co-induction of bimoclomol observed previously. These findings indicate that bimoclomol may be of value in targeting HSF-1 so as to induce up-regulation of protective Hsp-s in a non-stressful manner and for therapeutic benefit.

Keywords: Chaperones; Hsp47; Hsp70; Hsp90; HSF-1; Bimoclomol

Cellular stress induces elevated levels of members of the heat shock protein family, including Hsp70 and Hsp90, via the heat shock factor-1 (HSF-1) pathway. Upon stress, for example heat shock, the inactive monomeric form of the HSF-1 transcription factor is converted to a DNA-binding homotrimer, which binds to the heat shock response element (HSE) found in the heat shock gene promoters. After trimerization, HSF-1 is phosphorylated, thereby stimulating its transactivation ability and causing expression of the heat shock protein genes [1].

Bimoclomol (/*R*,*S*/-*N*-[2-hydroxy-3-(1-piperidinyl) propoxy]-3 pyridine carboximidoyl-chloride maleate, Biorex R&D, Hungary) is a non-toxic hydroxylamine derivative, which has been shown to have a wide variety of therapeutic effects as well as having potential therapeutic uses in the treatment of diabetes including diabetic retinopathy [2] and diabetic peripheral neuropathies [3], cardiac dysfunction [4,5], and cerebrovascular disorders [6]. It has also been shown that in the presence of bimoclomol, under conditions of stress, elevated levels of various stress proteins, such as Hsp60, Hsp70, Hsp90,

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☆☆ *Abbreviations:* HSE, heat shock element; HSF-1, heat shock factor-1; Hsp47, 47 kDa heat shock protein; Hsp70, 70 kDa heat shock protein; Hsp90, 90 kDa heat shock protein.
* Corresponding author. Fax: +36-1-266-7480.
E-mail address: csermely@puskin.sote.hu (P. Csermely).
[1] Present address: Department of Immunopharmacology, Institute of Drug Research, Budapest, Hungary.

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and Grp94, are produced compared to the levels observed with stress alone [7].

Elevated levels of Hsp-s have been shown to have protective effects. There is a clear potential therapeutic value of causing increased expression of the heat shock proteins, particularly in individuals suffering from cerebral or cardiac ischemia and neurodegenerative diseases [8–10]. Therefore, the use of a non-toxic drug, such as bimoclomol, may be of therapeutic importance. This paper identifies the mechanism how bimoclomol augments the induction of heat shock proteins after a cellular stress by showing that bimoclomol induces a prolonged binding of HSF-1 to the respective DNA elements.

Materials and methods

Cell culture. HSF*wt*, HSF-1 null (HSF −/−) mouse fibroblast cells [11,12] were cultured in Dulbecco's modified Eagle's medium. L929 mouse fibroblast cells were grown in modified Eagle's medium. K562 human erythroleukemia and Wehi-164 mouse fibroblast cells were cultured in RPMI-1640 medium. To all media 2 mM L-glutamine, 1% v/v penicillin–streptomycin (10,000 IU/ml), and 10% fetal bovine serum were added. The media of the HSF*wt* and HSF −/− cells also contained 1× non-essential amino acids, 0.11 g/L sodium pyruvate, pyridoxine, and 0.005% β-mercaptoethanol. Cell culture reagents were supplied by Gibco and Sigma.

Measurement of Hsp70 mRNA decay. For RNA preparations, L929 cells were plated two days before treatment into tissue culture dishes at densities resulting in 10^7 cells per sample at the time of treatment. Heat stress was administered by immersing the culture vessels into a 42 °C water bath for 30 min, after which the cells were placed into a 37 °C thermostat for recovery. Total cellular RNA was prepared after 0.5–6 h of recovery using RNeasy midi spin columns according to the manufacturer's instructions (Qiagen). The RNA was quantified spectrophotometrically at 260 nm and the integrity of the samples was analyzed by agarose gel electrophoresis. For cDNA preparation, random hexamer primers (Amersham–Pharmacia) and M-MLV reverse transcriptase or SuperScript II RT were used according to protocols described by Gibco in Supershift Preamplification System. PCRs were performed in a 50 μl final volume, using a Perkin–Elmer Thermocycler. Reactions contained 2–5 μl ss cDNA from the first strand reaction, 1× RT buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 200 nM of specific HPLC-purified forward and reverse primers (actin F: 5′-AGCTGAGAGGGAAATCGTGC, actin R: 5′-GATGGAGGG GCCGGACTCAT, Hsp70 F: 5′-CGGCTAGAGCAGGTACCACG A, Hsp70 R1: 5′-AGCACCATGGACGAGATCTCC, and Hsp70 R2: 5′-GTCCAGCCCGTAGGCGATG), and 1 U of *Taq* DNA polymerase. Labeling of the DNA was performed by incorporating 0.5–1 μCi [α-^{32}P]dCTP into the reaction mixtures. Amplification was performed using hot start (3 min at 94 °C), followed by 30–40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 74 °C. To facilitate quantitative analysis, samples were removed from the PCRs after different numbers of cycles of amplification, as indicated in the figure legends. PCR products were analyzed by agarose gel electrophoresis. Gel photographs were scanned for quantitative analysis using the Advanced American Biotechnology ID program. Gels containing radiolabeled PCR products were air-dried and autoradiographed using X-ray films or phosphorimager.

Measurement of Hsp70 decay. Stability of Hsp70 protein was measured by a competitive ELISA developed at Biorex. Briefly, high binding polystyrene plates (Corning Costar) were coated with anti-rabbit IgG (Sigma, 5 μg/ml in 70 mM carbonate buffer, pH 9.0), then after blocking with BSA (10 mg/ml, in PBS) affinity purified Hsp70-specific polyclonal antibodies, recognizing mouse, rat, and human Hsp70-s [13], were added. After washing (25 mM Tris, pH 8.0 containing 0.2% Tween 20), standards (purified recombinant Hsp70 at different concentrations to create standard curve) and samples (cell lysates) were added to the wells and the plates were pre-incubated at 37 °C for 30 min. Then, competitor (biotin labeled recombinant Hsp70 protein) was added to the wells and the plates were incubated at room temperature for 60 min. After washing, alkaline phosphatase conjugated streptavidin was added to determine the amount of bound biotinylated antigen. Enzyme activity was read by an iEMS plate reader (Labsystems). Cell lysates for the ELISA were prepared by freezing, thawing, and sonicating the cells in a buffer containing 50 mM Tris and 5 mM EDTA, pH 7.48.

Measurement of cytoprotection. HSF*wt* and HSF −/− cells [11,12] were seeded in a 96-well tissue culture plate at 10^4 cells/well density in 100 μl culture medium. After 6–8 h the medium was changed to serum-free medium and the cells were further incubated at 37 °C for 24 h. Then, 10 μl of 5 mg/ml MTT solution (Thiazolyl Blue, Sigma) was added to the wells and the cells were further incubated at 37 °C for 4 h. The developed formazan crystals were dissolved in 100 μl of 10% SDS solution and the absorbance was measured at 620 nm by a plate reader (Labsystems) after a 4-h incubation period.

Binding of [^{3}H]bimoclomol to HSF-1, Hsp70, and Hsp90. Equilibrium dialysis was used to determine the binding of bimoclomol to different proteins. Chamber A contained the mixture of 8 μM protein and 2.1 nM (2700 cpm/50 μl sample) [^{3}H]bimoclomol (Institute of Isotopes, Hungary) in a buffer of 10 mM Hepes (pH 7.35), 20 mM NaCl, and 1 mM EDTA, while chamber B contained the buffer alone. The two chambers were separated by a Spectra/Pore Biotech dialysis membrane (MWCO: 15,000). After equilibration (24 h at 37 °C), isotope content in both chambers was determined by liquid scintillation. Total counts measured in chambers A and B were normalized to control and then bound fraction (Fb) was calculated as described by Rivory et al. [14].

For the preparation of recombinant HSF-1, an *Nco*I site was incorporated at the initiation codon of the human HSF-1 cDNA clone (generous gift of Carl Wu (NIH, USA)) by PCR directed mutagenesis (5′-TAT**CCATGG**ATCTGCCCGTGGGCC). Then, HSF-1 cDNA was cloned into the *Nco*I–*Bam*HI sites of pET11d and expressed in *Escherichia coli* BL21(DE3) strain (Novagen) as described by Rabindran et al. [15]. HSF-1 was purified as described by Soncin et al. [16]. Native human HSF-1 was purified by a DNA-affinity resin [17,18] from K562 human erythroleukemia cells. Rat Hsp70 was cloned from heat shocked heart mRNA using pBluescript and *Eco*RV insertion with overhanging T-tags as described [13]. Hsp90 was purified from rat liver to homogeneity as described previously [19]. α-Acidic-glycoprotein (AGP) and BSA were from Sigma.

Phosphorylation of HSF-1. The phosphorylation status of HSF-1 was assessed by its characteristic supershift on SDS–PAGE. After being exposed to heat shock, K562 cells were pelleted at 2000*g* for 2 min and the supernatants were removed. Pellets were frozen in dry ice/methanol and thawed and the cells were resuspended in lysis buffer (20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) and SDS–PAGE and protein blotting was carried out using a Bio-Rad gel electrophoresis unit and a semi-dry transfer apparatus onto an NC membrane (Schleicher & Schuell, Protran BA85). Detection was by a rabbit anti-HSF-1 antibody (a generous gift of Richard I. Morimoto) followed by a horseradish-peroxidase-conjugated secondary antibody (Promega).

Gel retardation assays. Binding reactions were performed using a specific synthetic HSE oligonucleotide (5′-GGACCCTGGAATATTC CCGATGCGG) (Sigma). Double stranded oligonucleotides were labeled at the 5′ end with [γ-^{32}P]ATP (Institute of Isotopes, Hungary) using T4 polynucleotide kinase (New England Biolabs). Whole cell

extracts (10 μg) were mixed with 0.25 ng of labeled HSE-containing oligonucleotide and 0.5 μg of poly(dI–dC) (ICN Biomedicals) in binding buffer (50 mM Tris, pH 7.8, 250 mM NaCl, 2.5 mM EDTA, and 25% glycerol) in a final volume of 25 μl. Binding reaction was performed for 20 min at room temperature. Samples were then electrophoresed on a non-denaturing 4% polyacrylamide gel in 1× Tnana buffer (6.7 mM Tris, pH 7.5, 3.3 mM Na-acetate, and 1 mM EDTA) at 4 °C at 10 V/cm. Gels were dried and exposed to X-ray films (Eastman Kodak Company) at −80 °C with intensifying screens. HSE binding activity was quantified by scanning the autoradiograms with a densitometer (Bio-Rad). For some experiments, nuclear extracts were prepared from cell lysates prior to EMSA [20]. The specificity of the binding was analyzed by competition with 100-fold excess of unlabeled HSE or mutant HSE oligonucleotides (5′-GGACCCTGGTTTAAAC CCGATGCGG) (Sigma).

Results and discussion

Bimoclomol does not effect the stability of Hsp70 or its mRNA

Vigh et al. [7] had previously observed a 1.2–2.5-fold increase in the level of several heat shock proteins such as Hsp60, Hsp70, Hsp90, and Grp94 as well as in Hsp70 promoter activity when cells were pre-treated with bimoclomol prior to heat shock in comparison with heat shock alone. Evidently, one manner in which bimoclomol could increase the amount of heat shock proteins is to stabilize heat shock proteins or their mRNAs. Heat shock is known to stabilize Hsp70 mRNA [21] therefore we examined if bimoclomol produces a similar effect. The parallel curves of Hsp70 mRNA decay of heat shocked cells (Fig. 1A) in the presence and absence of bimoclomol show that the drug does not have a significant effect on Hsp70 mRNA stability. Similarly, we observed no differences in the decrease of Hsp70 levels, which indicated that bimoclomol leaves Hsp70 protein stability unchanged as well (Fig. 1B).

Bimoclomol acts via HSF-1

To determine the potential involvement of HSF-1 in the chaperone co-inducing effects of bimoclomol, constructs containing the full-length promoter regions of either Hsp90β (referred to as Hsp90βL) or Hsp47 were transiently transfected into wild-type mouse fibroblasts (HSF*wt*) or into fibroblast cells from HSF-1 knockout mice (HSF −/− cells [11,12]). In agreement with our previous data [7] treatment with heat shock enhanced promoter activity of both Hsp90 and Hsp47 approximately 4- and 2-fold, respectively. However, cells pretreated with bimoclomol prior to heat shock showed an approximately 2-fold further enhancement in Hsp90βL promoter activity. On the contrary, there was no increase in promoter activity when HSF −/− cells were treated with bimoclomol or heat shock or a combination

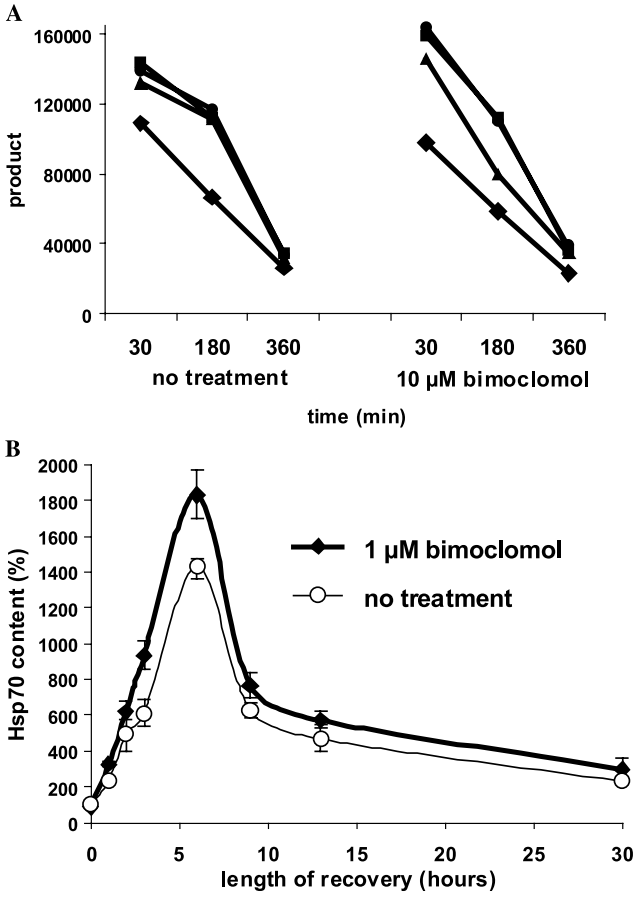

Fig. 1. Effect of bimoclomol on Hsp70 mRNA and protein decay. (A) The gradual decrease of the amount of Hsp70 mRNA after heat shock. mRNA levels were determined using RT-PCR as described in Materials and methods. The RNA samples used as template in these RT-PCRs were extracted after 30, 180, and 360 min of recovery from cells that were heat stressed (30 min, 42 °C) in the absence (left on the graph) or in the presence (right on the graph) of 10 μM bimoclomol. The four curves in both sets of samples represent aliquots of PCR samples taken after 33, 35, 37, and 39 cycles of amplification. Data are representative of three experiments. (B) Effect of bimoclomol on Hsp70 protein decay. Wehi-164 cells were treated with 10 μM bimoclomol and 30 min later the cells were exposed to heat shock at 43 °C for 30 min. Then, cells were allowed to recover for the indicated length of time, cell lysates were prepared, and Hsp70 content was determined by an Hsp70 ELISA as described in Materials and methods. No difference was observed in the kinetics of Hsp70 decay between the bimoclomol-treated and the untreated samples. Results are expressed as means ± SEM of four parallels of a representative experiment.

of both bimoclomol and heat shock. Similarly, no elevation of either Hsp70 or Hsp90 protein level was observed with any of the treatments in HSF −/− cells (data not shown).

We wanted to know if the inability of bimoclomol to induce heat shock proteins in HSF −/− cells also blocks its cytoprotective activity. HSF*wt* and HSF −/− cells were simultaneously treated with bimoclomol and deprived of serum for 24 h and their viability was measured by the MTT assay. While bimoclomol significantly increased the survival of HSF*wt* cells it did not influence

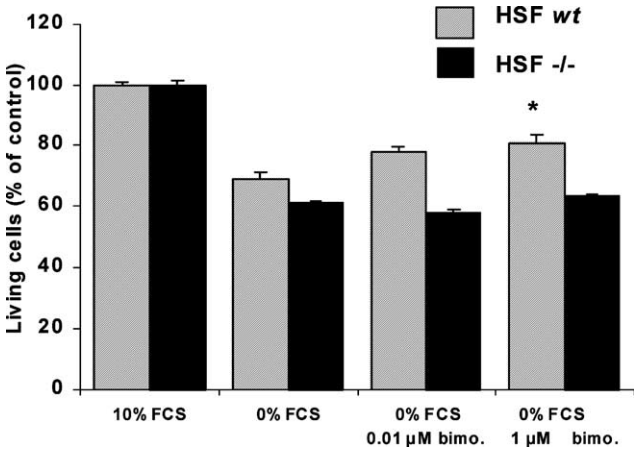

Fig. 2. Bimoclomol cannot exert its cytoprotective effect on HSF −/− cells. HSF*wt* and HSF −/− cells were grown in serum-free medium for 24 h with bimoclomol present during the whole length of time. The amount of living cells was determined by MTT assay. Growth in serum-free medium resulted in a 31% or 40% decrease in viability for the wild-type and the knockout cells, respectively. In the most effective concentration, 1 μM bimoclomol significantly increased ($*p < 0.01$) HSF*wt* cell-viability from approximately 70–80%. No change was observed in the viability of HSF1 −/− cells upon bimoclomol treatment. Results are expressed as means ± SEM of 10 parallels of a representative experiment.

the survival of HSF −/− cells (Fig. 2). Since serum deprivation is known to induce the HSF response in various cells [22,23], and bimoclomol induces an enhanced synthesis of Hsp70 after serum deprivation [23], our data strongly suggest that bimoclomol mediates both its heat shock promoter co-stimulatory and cytoprotective effects through HSF-1.

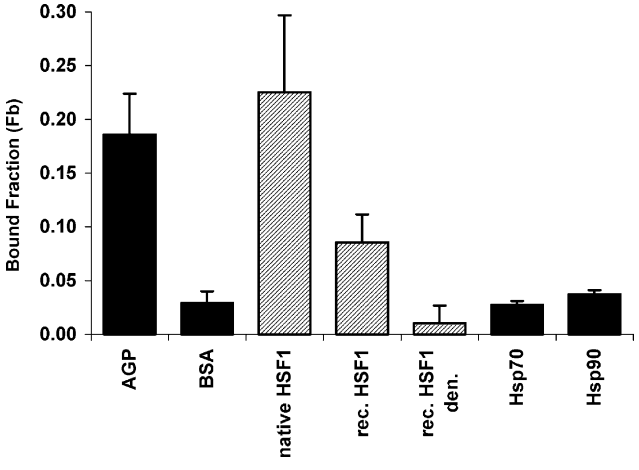

Fig. 3. Bimoclomol binds to HSF-1. [^{3}H]Bimoclomol (2.1 nM) was added to 8 μM of α-acidic-glycoprotein (AGP), BSA, native HSF-1, recombinant human HSF-1, heat denatured HSF-1 (80 °C for 5 min), recombinant Hsp70 or purified Hsp90. Equilibrium dialysis was performed as described in Materials and methods. Data are means ± SD of three independent experiments.

Binding of [^{3}H]bimoclomol to HSF-1

To address the question, if the major constituents of the HSF-1 complex, Hsp70, Hsp90, and HSF-1 itself [1] may be direct targets of bimoclomol, we examined the binding of radiolabeled, [^{3}H]bimoclomol to purified proteins using equilibrium dialysis. Neither Hsp70 nor Hsp90 bound a significant amount of [^{3}H]bimoclomol. However, binding of bimoclomol to both recombinant and native HSF-1 was observed (Fig. 3). AGP served as a positive control since it was known to bind bimoclomol [23] and BSA was used as a negative control. No binding of [^{3}H]bimoclomol to heat-denatured HSF-1 was detected. Direct binding of bimoclomol to HSF-1, or its complexes with various Hsp-s, may contribute to the action of bimoclomol.

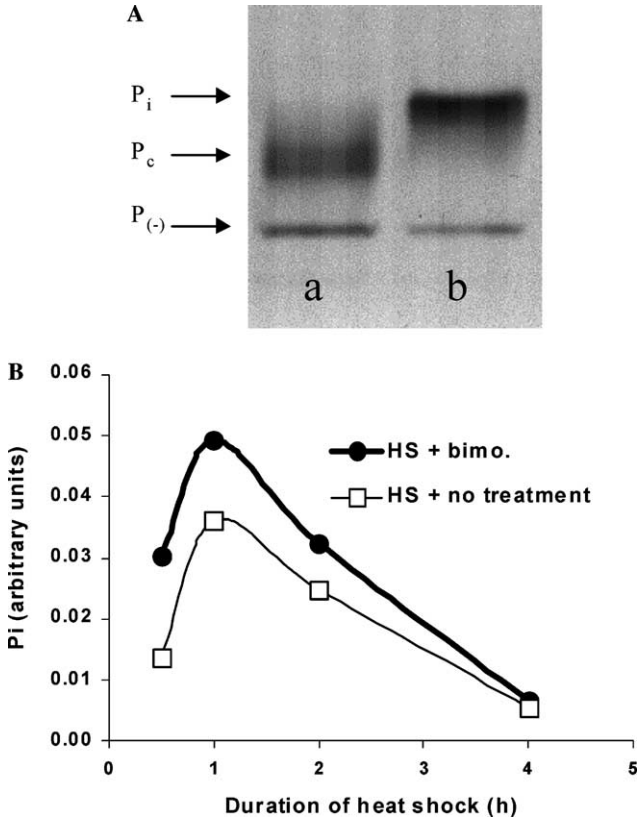

Fig. 4. Effect of bimoclomol on the phosphorylation of HSF-1. Densitogram of a typical blot is shown in (A). The inducible phosphorylated form (P_i) of human HSF-1 was detected at approximately 95 kDa, the constitutively phosphorylated form (P_c) of the protein appeared at approximately 85 kDa, and the polypeptide band at 76 kDa represented the dephosphorylated form ($P_{(-)}$) of HSF-1. Lanes (a) and (b) show extracts from control and heat shocked cells, respectively. (B) The graphical representation of the phosphorylation of HSF-1 in the absence (open symbols) or presence (filled symbols) of 5 μM bimoclomol in control K562 cells (37 °C) or after continuous heat shock at 42 °C for various lengths of times, as indicated. Bimoclomol was added to the cells 30 min before the beginning of heat shock and the amount of the hyperphosphorylated HSF-1 (P_i) was assessed by its characteristic supershift. The result of a representative experiment is shown.

Bimoclomol moderately increases the phosphorylation of HSF-1

To assess the effect of bimoclomol on the late phase of HSF-1 activation, HSF-1 phosphorylation was monitored by measuring the characteristic phosphorylation-induced supershift of HSF-1 on Western blots (Fig. 4A). Heat shock treatment of K562 cells for 0.5, 1, 2, and 4 h at 42 °C gradually increased the inducibly phosphorylated form up to 1 h, after which the amount of the phosphorylated form of HSF-1 began to decrease. Pre-treatment with bimoclomol moderately increased the amount of inducibly phosphorylated HSF-1 (Fig. 4B).

Bimoclomol prolongs the binding of a specific complex to HSE

The weak binding of bimoclomol to purified native and recombinant HSF-1 (Fig. 3) raised the possibility that bimoclomol modulates HSF-1 DNA-binding in cells. To test this hypothesis, K562 cells were treated with either bimoclomol, heat shock or a combination of both bimoclomol and heat shock prior to the preparation of whole cell extracts to determine if samples treated with both bimoclomol and heat shock showed enhanced binding to a labeled oligonucleotide carrying an Hsp90 heat shock response element.

The addition of the nuclear extract to labeled HSE-containing oligonucleotides resulted in the formation of a specific complex (Fig. 5A), as indicated by the arrows. This complex was readily competed away by excess unlabeled oligonucleotide carrying the HSE element. Nuclear extracts of HSF −/− cells treated with bimoclomol, heat shock or a combination of both bimoclomol and heat shock showed no specific complex binding to the labeled HSE (data not shown). These data would indicate that, as no specific complex was observed in HSF −/− cells, HSF-1 is a major constituent of the complex or is required to form the complex. This was confirmed by the addition of an HSF-1-specific antibody to the assay that resulted in the formation of a supershifted complex of low electrophoretic mobility with the HSF*wt* cells (data not shown).

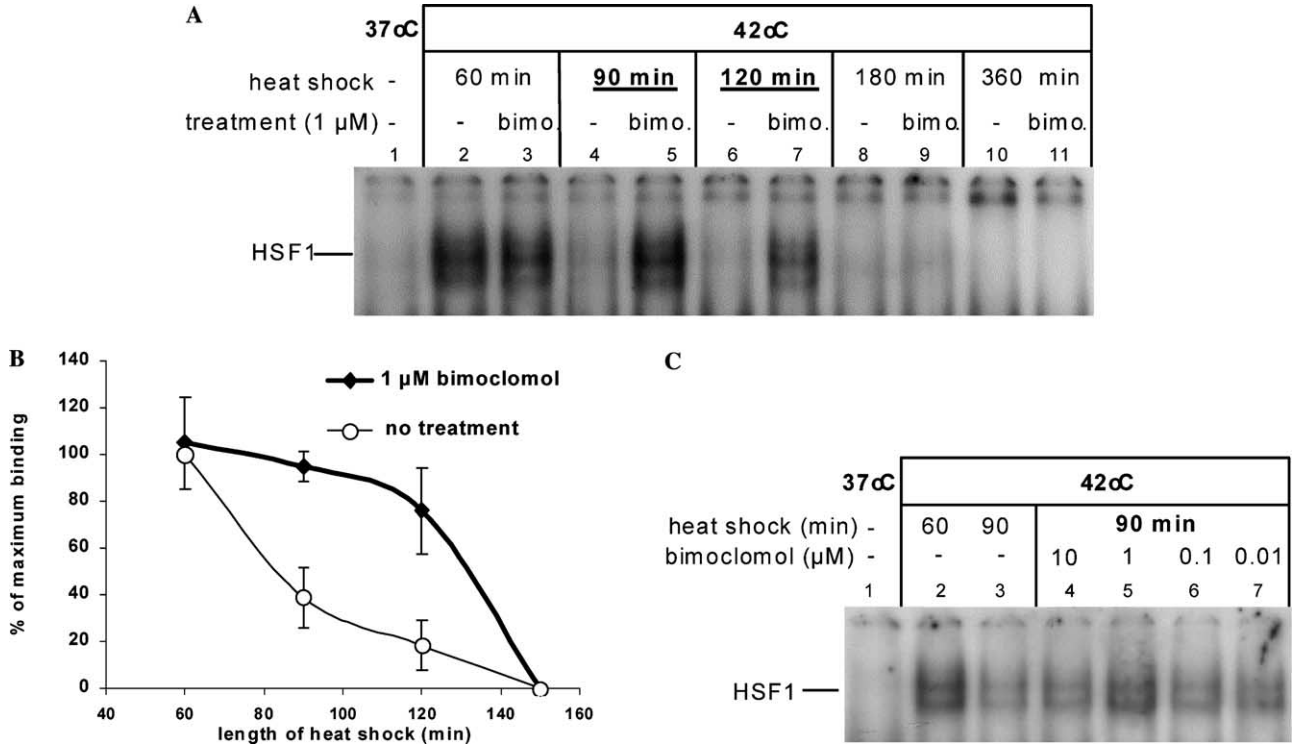

Fig. 5. Bimoclomol prolongs the binding of a specific complex to a labeled HSE. K562 cells were treated 30 min before being exposed to continuous heat shock at 42 °C for the indicated length of time, without a recovery period. Then, whole cell lysates were prepared and 10 μg extracts were incubated with a labeled Hsp90 HSE under conditions described in the methods section. Free and bound DNAs were resolved on a 4% polyacrylamide gel and visualized by autoradiography. (A) Bimoclomol does not have any effect at early time points of the heat shock response (0–30 min, data not shown) or in case of maximal HSF-1 binding activity (lanes 2–3). However, bimoclomol extends HSF-1 binding activity to approximately 120 min (lanes 4–5 and 6–7). This effect is no longer detectable at 150 min or later. A representative experiment is shown. (B) Numerical representation of the HSE binding. The intensity of the binding of the HSF-1-specific complex of the untreated sample at 60 min was designated 100%. Data are means ± SEM of $n = 2$–5 experiments. (C) concentration-dependent effect of bimoclomol on the DNA-binding of HSF-1. The maximal effect is observed at 1 μM bimoclomol treatment (lanes 3 and 5).

Bimoclomol induced a significant prolongation of the binding of HSF-1 to HSE (Figs. 5A and B). The concentration dependence of the effect is demonstrated in Fig. 5C. This effect is in agreement with both the moderately enhanced phosphorylation of HSF-1 and the heat shock protein co-induction after bimoclomol treatment. A prolonged binding of HSF-1 probably induces a more extended transcription of Hsp genes. However, in the absence of any original stress, HSF-1 does not bind to the HSE. The inability of bimoclomol to prolong DNA-binding under these conditions is in good agreement with its ineffectiveness to induce any Hsp synthesis in the absence of stress.

In summary, our findings demonstrated that bimoclomol mediates its co-inducer effect on heat shock protein gene expression and cytoprotective action via HSF-1. Although further studies are required to examine the precise manner in which bimoclomol exerts its effect on HSF-1 or its complexes with heat shock proteins, this appears to involve a moderately enhanced phosphorylation of HSF-1 and prolonged binding of HSF-1 to DNA. Bimoclomol-induced fluidization of cellular membranes [24] may also lower the threshold of the heat shock response, and thus contribute to the chaperone co-inductive property [7] of this drug. Currently we do not know how membrane fluidization is linked to the activation of HSF-1. Bimoclomol does not influence protein denaturation in cells [24]. However, very effectively, bimoclomol may use both a lipid-mediated signal leading to an enhanced HSF-1 phosphorylation as well as a prolonged binding of HSF-1 to the heat shock elements to enhance heat shock protein induction in stressed cells.

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