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# Hypoxia-inducible factor 1α is deregulated by the serum of rats with adjuvant-induced arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is known to be associated with increased risks of hypoxia-related diseases, whose progresses are critically determined by HIF-1α. The authors hypothesized that the hypoxia-related complications of RA are associated with HIF-1α deregulation by some factor(s) in RA serum. Arthritis was induced in female Lewis rats by injecting complete Freund's adjuvant. The effects of arthritic rat serum (ARS) on hypoxic responses were investigated by incubating Hep3B cells in ARS. In the presence of ARS, HIF-1α was down-regulated and inactivated under hypoxic conditions. ARS inactivated AKT and mTOR, which led to impaired HIF-1α protein synthesis. Furthermore, insulin was found to be deficient in ARS and insulin supplementation fully recovered HIF-1α synthesis with AKT and mTOR activation. These results suggest that HIF-1α deregulation by components in serum is responsible for the RA-associated aggravation of hypoxic diseases in extra-articular tissues.



Although rheumatoid arthritis (RA) mainly manifests joint inflammation [1], it is also known to elevate the risks of other diseases. For example, a prospective cohort study showed that the incidence of myocardial infarction is 2.8-fold higher in women with RA [2]. RA is also known to induce hypoproliferative anemia by decreasing erythropoietin (EPO) production in response to hypoxia [3].

Hypoxia-inducible factor-1α (HIF-1α) is now viewed as a master molecule that directs hypoxic responses by transactivating more than 60 genes [4]. Under normoxic conditions, HIF-1α is degraded by sequential reactions of prolyl-hydroxylation, pVHL binding, ubiquitination, and proteasomal proteolysis. The prolyl-hydroxylation is limited under hypoxic conditions, and thus HIF-1α becomes stable [5]. Also, growth factors and cytokines up-regulate HIF-1α by increasing the synthesis of HIF-1α protein [6]. In particular, the PI3K/AKT/mTOR signaling cascade plays a central role in the translation of HIF-1α. Furthermore, growth factors, such as, insulin, IGF-1, and EGF, have been reported to induce HIF-1α by stimulating this signaling through their tyrosine-kinase receptors [7,8].

Recently, HIF-1α was identified to be a key player in the pathogenesis of RA and to be a potential therapeutic target in RA [9]. HIF-1α is expressed at higher levels in the synovial tissues of RA patients than in those of fracture patients [10]. Furthermore, loss of HIF-1α prevented the joint swelling and cartilage destruction observed in wild-type mice [11]. Summarizing, intra-articular hypoxia and pro-inflammatory cytokine production induce HIF-1α, which in turn aggravates angiogenesis and joint inflammation. However, it should be noted that HIF-1α expression and function have been investigated only as local events in inflamed joints, and that as yet, the effects of RA on HIF-1α-mediated hypoxic responses have not been reported in extra-articular tissues.

As mentioned above, RA aggravates hypoxia-related diseases and the progresses of these diseases are known to be critically determined by HIF-1α. Given this, we speculated that the hypoxia-related complications of RA are associated with impaired HIF-1α response to hypoxia. More specifically, our hypothesis was that some serum factor produced during RA interferes with HIF-1α-mediated hypoxic adaptation in extra-articular tissues. To test this hypothesis, we examined whether arthritic rat serum (ARS) affects HIF-1α expression and activity under hypoxic conditions. Although this arthritis model does not directly reflect clinical RA conditions, we believe that it provides new perspectives concerning the pathogenesis of the hypoxia-related, extra-articular complications of RA.

## Materials and methods

*Adjuvant-induced arthritis*. Animal experiments were performed in accord with the Seoul National University Laboratory Animal Maintenance Manual. Arthritis was induced using an adjuvant, as

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described previously [12]. Briefly, Female Lewis rats (aged 8 weeks) were divided into two groups. The first group (normal rats) was a control group left untreated. The second group (arthritic rats) was subcutaneously injected at the tail base and four soles with 0.1 ml of complete Freund's adjuvant (H37 Ra) provided from DIFCO (Detroit, MI). Two days later, rats were re-injected with the same adjuvant solution. Fourteen days after the first vaccination, both normal and arthritic rats were fasted for 8 h to avoid any feeding-associated changes in serum factors, and then sacrificed for blood sampling and to determine the degree of arthritis. Joint inflammation severities were determined by measuring increases in distal hind leg volumes [12]. Blood samples were centrifuged to obtain clean sera, and the sera were stored at −70 °C.

*Cell culture and hypoxic induction*. Hep3B cells were cultured in α-modified Eagle's medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal calf serum. To examine the effects of rat sera on hypoxic responses, Hep3B cells were cultured in a serum-free media for 16 h, and then incubated in media containing 10% of normal or arthritic rat sera 4 h before hypoxic incubation.

*Semiquantitative RT-PCR*. EPO and VEGF mRNA levels were determined by semiquantitative RT-PCR. After one cycle of reverse transcription at 48 °C for 30 min, cDNAs were amplified with 5 μCi [α-32P]dCTP over 18–25 amplification cycles of 94 °C/53 °C/70 °C. The PCR fragments were electrophoresed in 4% polyacrylamide gels and autoradiographed. The primers used were designed as described previously [13].

*Western blot analysis*. Cell extracts were electrophoresed in 8–10% SDS/polyacrylamide gels and transferred to Immobilon-P membranes, which were incubated overnight 4 °C with primary antibodies, such as anti-HIF-1α [14], anti-AKT (Cell Signaling, Beverly, MA), anti-phospho-AKT (Cell Signaling), anti-mTOR (Cell Signaling), anti-phospho-mTOR (Cell Signaling), or anti-β-tubulin (SantaCruz), in 5% nonfat milk. Horseradish peroxidase-conjugated antibody (SantaCruz) was used as a secondary antibody and immune complexes were visualized using enhanced chemiluminescence plus kits (GE-Healthcare, Piscataway, NJ).

*Reporter assay*. An EPO-enhancer-driven luciferase reporter gene was constructed, as described previously [14]. To evaluate the translational rate of HIF-1α mRNA, we cloned the 5′-UTR (1–284) segment of HIF-1α mRNA using RT-PCR, and then inserted the cloned DNA between thymidine kinase promoter and luciferase in GL3 promoter plasmid. Hep3B cells were co-transfected with 1 μg each of luciferase reporter plasmid and the β-galactosidase plasmid using Lipofectamine (Invitrogen). After stabilizing, the cells were incubated for 16 h in 20% or 1% $O_2$, and luciferase activities were determined using a luminometer. Differences in transfection efficiencies and sample preparations were corrected by normalizing luciferase activities versus β-galactosidase activities.

*Immunoenzymometric assays*. ELISA kits for rat IGF-1, IGFBP-3, and insulin were purchased from Immunodiagnostic System (Fountain Hills, AZ), Mediagnost (Reutlingen, Germany), and LINCO Research (St. Charles, MO), respectively. Assays were performed according to the manufacturer's instructions.

*Statistical analysis*. The Mann–Whitney *U*-test was used for two-group comparisons and ANOVA for linear regression analysis. Statistical tests were two-sided and differences were considered significant when *P* values were <0.05.

## Results

### *Induction of arthritis in rats*

On day 14 after the first vaccination, most ankles in the arthritis group were significantly swollen. The volumes (mean ± SD, $cm^3$) of right and left distal hind limbs were 3.65 ± 0.20 ($p < 0.01$ vs. control right) and 3.60 ± 0.25 ($p < 0.01$ vs. control left) in arthritic rats; corresponding control rat values were 2.35 ± 0.13 and 2.47 ± 0.23.

### *Effects of arthritic rat sera on HIF-1α-mediated, hypoxic gene induction*

We investigated the possibility that arthritic rat serum (ARS) contains a factor(s) responsible for HIF-1α suppression. Since Hep3B is known to induce EPO and VEGF sensitively in response to hypoxia, this cell line was chosen for this experiment. As was expected, EPO and VEGF mRNA levels increased under hypoxic conditions in 10% fetal bovine serum (Fig. 1A, 1–2 lanes). To examine the hypoxic gene induction in normal rat serum (NRS) or ARS, the cells were incubated in media containing 10% of sera obtained from normal ($n = 6$) or arthritic ($n = 6$) rats. Hypoxic levels of EPO and VEGF mRNAs were generally lower in ARS than in NRS (Fig. 1A, left panel). PCR band densities were quantified using ImageJ 1.36b image analysis software (NIH, USA), and ratios of EPO and VEGF to β-actin were found to be significantly reduced in cells exposed to ARS (Fig. 1A, right panel). Furthermore, it was also found that the hypoxic induction of HIF-1α was significantly attenuated in ARS versus that in NRS, whereas ARNT and β-tubulin levels were no different (Fig. 1B). We analyzed the transcriptional activity of HIF-1α using a luciferase reporter, and found that hypoxic reporter activation was significantly attenuated by ARS (Fig. 1C). Both EPO levels and reporter activities were found to be positively related ($p < 0.01$ by ANOVA) with HIF-1α levels (Fig. 1D), which further supports the notion that EPO expression and reporter activity depend on HIF-1α.

### *Effects of ARS on HIF-1α stability and synthesis*

To evaluate HIF-1α degradation, HIF-1α was stabilized in hypoxia and then destabilized by reoxygenation. Baseline HIF-1α levels were somewhat different, but HIF-1α degradation rates were not different (Fig. 2A). Next, HIF-1α synthesis was estimated in the presence of MG132 which blocks HIF-1α degradation. After MG132 treatment, HIF-1α levels in NRS noticeably accumulated in a time-dependent manner, but did not in ARS (Fig. 2B). This finding indicates that HIF-1α protein synthesis is blocked by ARS. To further evaluate the translation of HIF-1α mRNA, we constructed a reporter gene containing the 5′-UTR segment of HIF-1α mRNA [8]. The reporter structure is illustrated in the upper panel of Fig. 2C. Compared to cells incubated in serum-free medium, cells incubated in NRS showed a significant enhancement in reporter activity regardless of oxygen tension. However, reporter activity was significantly reduced in ARS (Fig. 3C). These results suggest that NRS can stimulate the translation of HIF-1α mRNA, but that ARS has no such ability.

### *Inhibition of the AKT/mTOR pathway in ARS*

Since the 5′-UTR-dependent regulation of HIF-1α translation is mediated by the AKT/mTOR pathway [6], we examined the effects of different sera on this signaling pathway. AKT and mTOR activation were estimated by measuring their phosphorylated forms. In ARS, both phospho-AKT and phospho-mTOR levels were significantly lower than those in NRS, whereas total levels of AKT and mTOR were not different (Fig. 3). These findings suggest that the impaired translation of HIF-1α in ARS is due to the inactivation of the AKT/mTOR pathway.

### *Serum factors responsible for HIF-1α translation*

Previous reports have demonstrated that serum growth factors stimulate HIF-1α translation via the PI3K/AKT/mTOR pathway

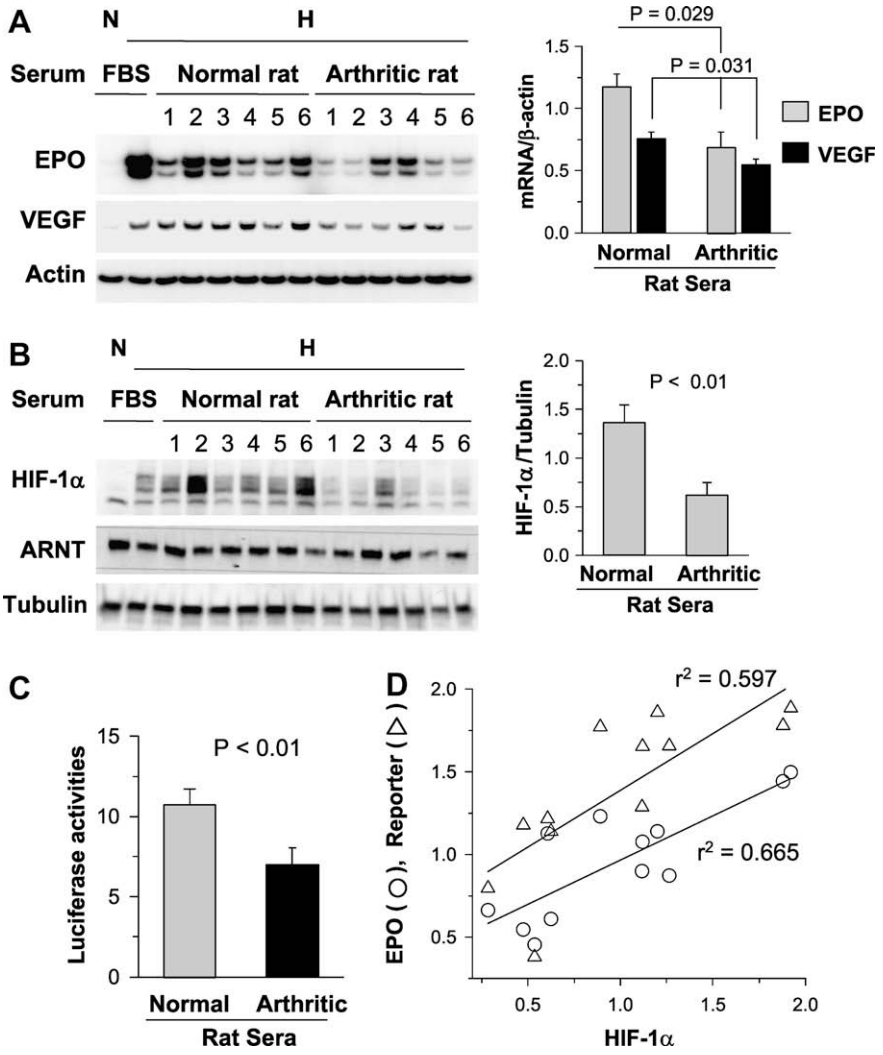


**Fig. 1.** The hypoxic inductions of HIF-1α and its downstream genes are blunted in ARS. (A) Decreased levels of EPO and VEGF mRNA in ARS. After 16 h serum-free incubation, Hep3B cells were cultured in media containing 10% of NRS ($n = 6$) or ARS ($n = 6$) 4 h prior to 16 h hypoxia. mRNA levels were determined by semiquantitative RT-PCR. NRS or ARS were numbered (1–6) for identification, and each number above a lane indicates that cells were incubated in the corresponding serum. Band intensities were quantified using ImageJ 1.36b software, and are presented as ratios versus β-actin. Means and SEs ($n = 6$) are plotted in the right panel. (B) Impaired HIF-1α expressiom in ARS. After 16 h of hypoxic incubation in the indicated media, proteins were analyzed by Western blotting (left panel). Numbers above lanes present the identification numbers of sera used. Protein band intensities were quantified using ImageJ, and are presented as ratios versus β-tubulin. Means and SEs ($n = 6$) are plotted in the right panel. (C) HIF-1 inactivation by ARS. After being co-transfected with EPO-enhancer luciferase and β-galactosidase plasmid, Hep3B cells were incubated in NRS or ARS under hypoxic conditions for 16 h. Luciferase activities are presented as ratios versus β-galactosidase activities; means and SEs ($n = 6$) are plotted. (D) Correlations between HIF-1α and EPO expression. EPO mRNA level ($n = 12$, circle) and reporter activity ($n = 12$, triangle) were plotted versus HIF-1α levels ($n = 12$); linear regression was analyzed using SigmaPlot.

[15,16], and thus, we checked insulin-like growth factor-1 (IGF-1) and IGF-binding protein-3 (IGFBP-3, a negative regulator of IGF-1) levels in rat sera. However, no differences in IGF-1 and IGFBP-3 levels were observed in NRS or ARS (Fig. 4A). We then checked insulin levels and found that the mean value of level in ARS were one third of that in NRS (Fig. 4A). To examine whether insulin deficiency in ARS is responsible for HIF-1α deregulation, we added recombinant insulin to ARS-containing medium. The insulin supplementation was found to recover the hypoxic induction of HIF-1α and to re-activate the AKT/mTOR pathway in a dose-dependent manner (Fig. 4B). Moreover, insulin rescued HIF-1α protein synthesis in ARS-containing medium (Fig. 4C). These results suggest that HIF-1α deregulation by ARS is responsible for insulin deficiency.

## Discussion

To determine which factors underlie the increased risk of hypoxia-related diseases in persons with RA, we hypothesized that such RA complications are associated with impaired HIF-1α response to hypoxia. The hypoxic induction and activation of HIF-1α were significantly attenuated in ARS. HIF-1α protein synthesis was impaired at the translational level, which may result from AKT and mTOR deactivation. Furthermore, of several serum growth factors capable of stimulating AKT, insulin was found to be significantly deficient in ARS, and insulin supplementation re-activated the AKT/mTOR pathway and recovered HIF-1α expression. These results suggest that RA-induced alterations in serum growth factors can influence HIF-mediated hypoxic responses in extra-articular tissues, which may provide an explanation for the hypoxia-related, extra-articular complications of RA.

To examine the effect of arthritic serum, we designed that fetal bovine serum was substituted with normal or arthritic rat serum in the culture medium. Even though primary rat cells were ideal for this study, the cells have drawbacks for mechanism study in that the efficiency of gene transfection is very low and more animals should be sacrificed for cell isolation. Therefore, we chose the

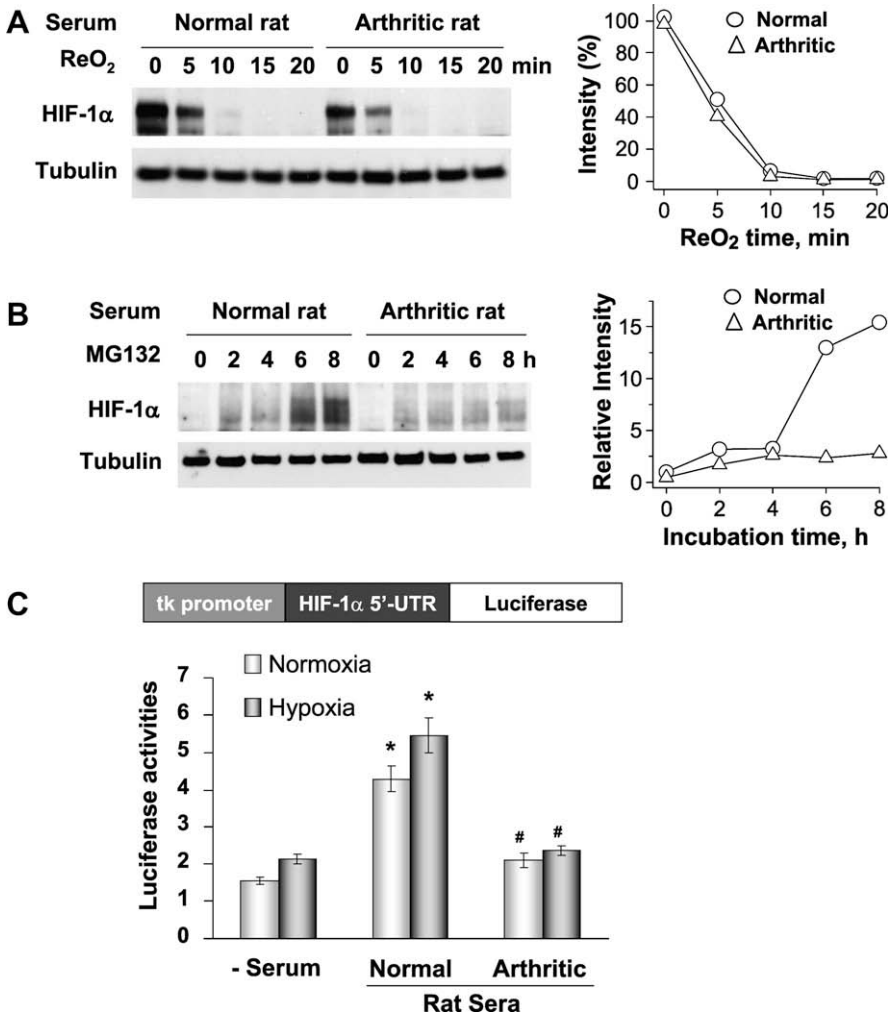


**Fig. 2.** ARS down-regulated HIF-1α at the translational level. (A) HIF-1α stability. Hep3B cells were cultured in the indicated sera for 2 h, incubated under hypoxic conditions for 8 h to express HIF-1α, and then re-oxygenated to induce HIF-1α degradation. HIF-1α levels were then analyzed by Western blotting and quantified using the ImageJ system. The time course of HIF-1α decay is plotted in the right panel. (B) HIF-1α synthesis. MG132 at 10 μM was added to serum-containing medium to prevent HIF-1α degradation. HIF-1α accumulation was monitored at the indicated time points by Western blotting and ImageJ analysis. The right panel shows the time course of HIF-1α accumulation in the different sera. (C) The 5′-UTR-dependent translation of HIF-1α mRNA. After being co-transfected with HIF-1α 5′-UTR/luciferase plasmid (the structure of the plasmid is illustrated above the figure) and β-gal plasmid, Hep3B cells were incubated in NRS or ARS-containing serum under hypoxic conditions for 16 h. Luciferase activities are presented as relative values versus β-galactosidase activity, and the means and SEs of the 4 experiments are plotted. $^{*}p < 0.05$ vs. cells cultured in serum-free medium; $^{\#}p < 0.05$ vs. cells cultured in NRS containing medium.

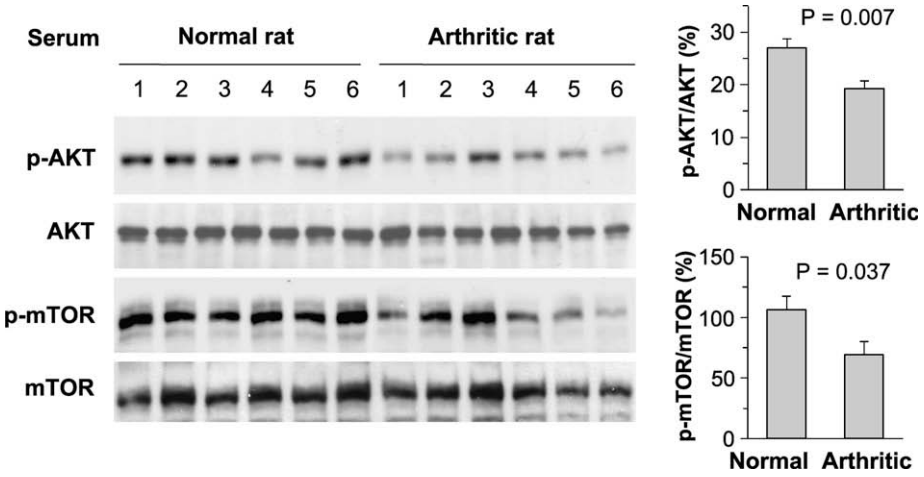


**Fig. 3.** AKT/mTOR signaling was inactivated in ARS. Hep3B cells were incubated in NRS or ARS-containing media under hypoxic conditions for 16 h. Numbers above lanes present the identification numbers of sera used. To evaluate the activations of AKT and mTOR, the ratios of phospho-forms to total forms were determined by Western blotting; results (means and SEs, $n = 6$) are plotted in the right panel.

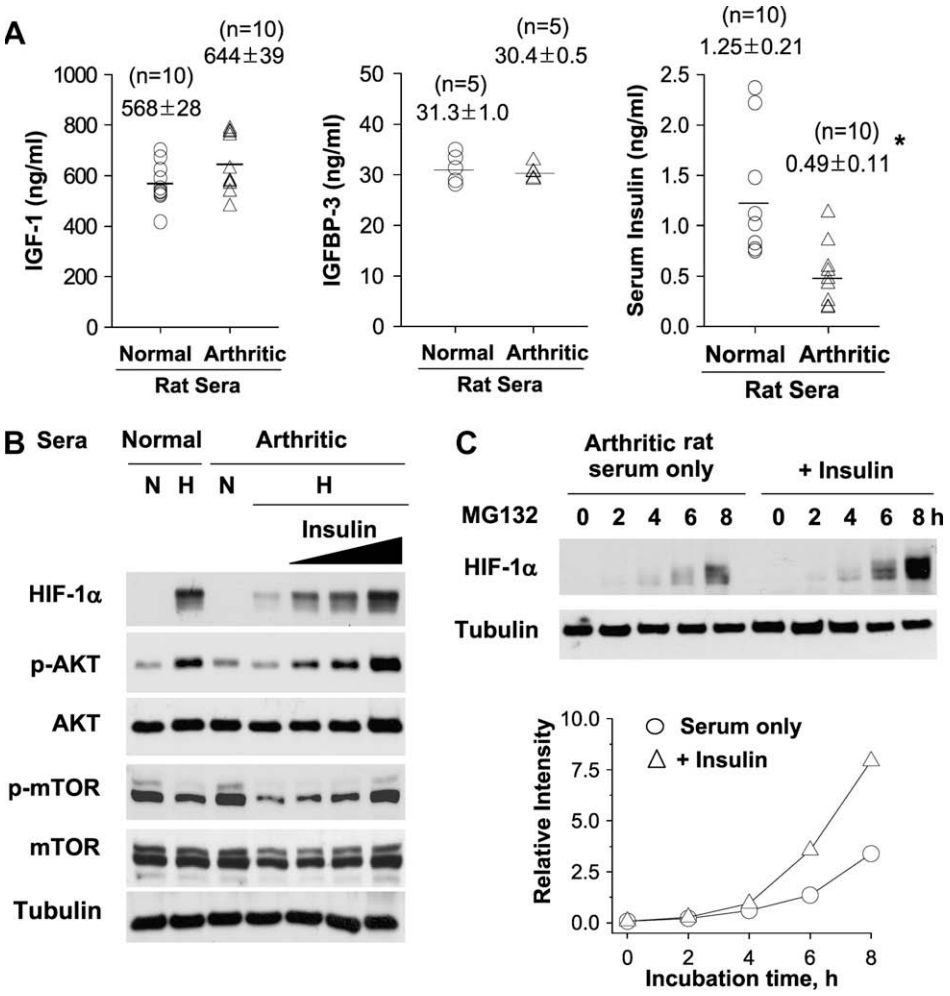


**Fig. 4.** Analyses of serum factor levels and HIF-1α recovery by insulin supplementation. (A) Rat IGF-1, IGFBP-3, and insulin levels in 5 or 10 sera per group were measured using ELISA kits. Results (means ± SEs) and sample numbers are inserted above the graphs. $^{*}p < 0.01$ vs. the non-arthritic group. (B) HIF-1α expression and AKT/mTOR activity recovery by insulin in Hep3B cells. Cells were incubated in NRS or ARS-containing media under hypoxic conditions for 16 h. They were then treated with recombinant insulin at 1, 10 or 100 nM for 1 h prior to hypoxic incubation. Indicated proteins were analyzed by Western blotting. (C) Insulin recovery of HIF-1α synthesis. Hep3B cells were treated with PBS or 1 nM insulin for 1 h and then treated with 10 μM MG132. HIF-1α accumulation was monitored at the indicated times by Western blotting and ImageJ analysis. The lower panel shows the time course of HIF-1α accumulation.

Hep3B cell-line in stead of primary cells. Then, is it reasonable that human cells are cultured in rat serum? Normally, to see some serum effects, after human cells were incubated in serum-free condition, the bovine or horse serum is added to the medium. In this respect, we though that rat sera can be also used for Hep3B culture to examine the arthritic serum effects.

HIF-1α is known to accumulate under hypoxic conditions due to the inhibition of PHDs [17,18]. Also, with respect to its post-transcriptional regulation, the 5′-UTR sequence in HIF-1α mRNA is targeted by the mTOR-mediated control of translation [7,8]. In ARS-containing medium, HIF-1α synthesis was severely inhibited, but its stability was not. This effect of ARS was also confirmed using a luciferase reporter containing the 5′-UTR of HIF-1α mRNA. In terms of the regulation of HIF-1α synthesis, various growth factors have been reported to activate the PI3K/AKT/mTOR signaling by interacting with their receptors and by so doing to stimulate HIF-1α synthesis [19]. In the ARS-containing medium, HIF-1α synthesis was retarded due to inactivation of this signaling pathway, which suggests that ARS is deficient in some growth factors. We hypothesized that IGF-1 and insulin might be responsible, because both are known to stimulate HIF-1α synthesis by activating the PI3K/AKT/mTOR pathway and are abundantly present in serum [16,20]. Our results show that insulin levels were severely depleted in ARS, whereas IGF-1 and IGFBP-3 levels were similar to those in control rat serum. However, it should be noted that we cannot rule out possible HIF-1α deregulatory contributions by other serum factors in ARS. Many pro-inflammatory cytokines are released from inflamed joints, and the productions of hormones and local tissue factors may be markedly altered during systemic inflammatory diseases like RA. More importantly, these factors have direct or indirect effects on PI3K signaling. Therefore, a more systemic approach would be useful for screening serum factors responsible for HIF-1α deregulation.

Clinically, changes in serum factor levels have been reported in RA patients. Matsumoto and Tsurumoto compared serum IGF-1 levels in RA and healthy women, and found that IGF-1 levels in RA serum were 33% lower than in normal serum [21]. They also analyzed IGFBP-3 levels in sera because IGFBP-3 interferes with the interaction between IGF-1 and its receptor. It was found that IGFBP-3 levels in RA serum were 190% higher than in normal serum, which might have further inhibited IGF-1 signaling. However, in the arthritis rat model, no changes were observed in IGF-1 or IGFBP-3 levels. On the other hand, serum insulin levels were found to be severely diminished in arthritic rats. In fact, it has been reported that insulin levels in rat serum falls at 10–15 days after adjuvant treatment in the arthritis rat model [22]. This report

supports our hypothesis that the observed insulin drop in serum is associated with HIF-1α deregulation in extra-articular tissues. However, since there have been no clinical reports analyzing the insulin levels during the acute phase of rheumatoid arthritis, it is uncertain whether the insulin reduction contributes to HIF-1α deregulation in RA patients. This is a limitation imposed by choosing a rodent RA model in the present study. Furthermore, although adjuvant-induced arthritis is a widely used model of RA, it might differ from human RA in terms of pathogenesis and disease progress. Thus, a similar experiment in human subjects might lead to more meaningful results and possibly to clinical implications.

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