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Evitar (L-Alanyl-L-Glutamine) Regulates Key Signaling Molecules in the Pathogenesis of Postoperative Tissue Fibrosis

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Abstract

Aims: Hypoxia and the resulting oxidative stress play a major role in postoperative tissue fibrosis. The objective of this study was to determine the effect of L-alanyl-L-glutamine (Ala-Gln) on key markers of postoperative tissue fibrosis: hypoxia-inducible factor (HIF) 1α and type I collagen. **Methods:** Primary cultures of human normal peritoneal fibroblasts (NPF) established from normal peritoneal tissue were treated with increasing doses of Ala-Gln (0, 1, 2, or 10 mM) with hypoxia ([2% O_2] 0-48 hours; continuous hypoxia) or after hypoxia (0.5, 1, 2, 4 hours) and restoration of normoxia (episodic hypoxia) with immediate treatment with Ala-Gln. Hypoxia-inducible factor 1α and type I collagen levels were determined by enzyme-linked immunosorbent assay. Data were analyzed with 1-way analysis of variance followed by Tukey tests with Bonferroni correction. **Results:** Hypoxia-inducible factor 1α and type I collagen levels increased in untreated controls by 3- to 4-fold in response to continuous and episodic hypoxia in human NPF. Under continuous hypoxia, HIF-1α and type I collagen levels were suppressed by Ala-Gln in a dose-dependent manner. L-alanyl-L-glutamine treatment after episodic hypoxia also suppressed HIF-1α and type I collagen levels for up to 24 hours for all doses and up to 48 hours at the highest dose, regardless of exposure time to hypoxia. **Conclusions:** L-alanyl-L-glutamine significantly suppressed hypoxia-induced levels of key tissue fibrosis (adhesion) phenotype markers under conditions of continuous as well as episodic hypoxia in vitro. This effect of glutamine on molecular events involved in the cellular response to insult or injury suggests potential therapeutic value for glutamine in the prevention of postoperative tissue fibrosis.

Keywords

type I collagen, HIF-1α, postoperative tissue fibrosis, adhesions, hypoxia, L-alanyl-L-glutamine

Introduction

Development of postoperative adhesions, which are a form of tissue fibrosis that occurs at sites of operative injury, is a near ubiquitous occurrence following surgical procedures at locations throughout the body.[1-4] The extent and severity of tissue fibrosis is commensurate with the magnitude of tissue injury. Peritoneal tissue fibrosis represents both de novo tissue fibrosis (adhesions) occurring at sites of operative procedures involving the peritoneum, such as myomectomies and ovarian cystectomies, and/or reformation of fibrotic tissue (adhesions) following adhesiolysis.[3,4]

At the sites of surgical procedures, a disruption of the vascular supply to the tissue results in a reduction in the availability of oxygen and nutrients. The consequence is a series of molecular biologic processes that attempt to mitigate the sequelae. These processes include recognition of low tissue oxygenation, which signals stabilization of HIF-1α. Upregulation of HIF-1α is associated with increases in levels of reactive oxygen and nitrogen species that result in a state of increased oxidative stress and trigger of the inflammatory cascade. HIF-1α activation induces changes in glucose metabolism that promote increased lactate production and reductive carboxylation of citrate for fatty acid production to fuel membrane synthesis in support of tissue growth.[5,6] Activation of HIF-1α also induces extracellular matrix remodeling and enhanced angiogenesis.[4,7,8] The sequelae of these changes include reduced fibrinolysis, fibroblast ingrowth and extracellular matrix deposition between adjacent tissue surfaces, leading to tissue fibrosis formation.[4,9,10] Subsequent vascularization establishes

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a means of resupplying oxygen and nutrients to surgically injured tissue.

Tissue hypoxia, resulting from vascular injury and loss during surgery, has been implicated in the development of tissue fibrosis through the induction of HIF-1α and various cytokines. These factors are secreted from stress-activated immune corpuscles in the peritoneum (milky spots).[11-13] They promote development of an "adhesion phenotype" in exposed fibroblasts,[14-16] a process also known as epithelial to mesenchymal transition.[17] A hallmark of this phenotypic transition is a shift in glucose metabolism that inhibits the Krebs cycle, generates lactate through anaerobic glycolysis, and promotes fatty acid synthesis to support tissue growth.[5] These changes stimulate the characteristic rise of profibrotic effectors secreted from milky spots, including transforming growth factor β (TGFβ), a potent autocrine stimulator of fibroblast activation, vascular endothelial growth factor, interleukins, fibronectin, and plasminogen activator inhibitor that promote unopposed fibrin deposition, neovascular growth, and ultimately, tissue fibrosis (adhesions).[7,18-20]

We have previously characterized the adhesion phenotype to manifest as an increase in basal levels of extracellular matrix proteins (such as types I and III collagens, α-smooth muscle actin, and fibronectin), growth factors, angiogenic factors, and reactive oxygen species.[8,15,16] The effect of oxygen-free radicals resulting from hypoxia can be reversed by superoxide dismutase (SOD), a powerful antioxidant. Indeed, in SOD-treated adhesion fibroblasts, suppression of adhesion phenotype marker (TGF-β1 and type I collagen) levels to those observed in normal peritoneal fibroblasts (NPFs) has been observed.[21] Further, we have previously demonstrated that scavenging superoxide ($O_2\bullet^-$) during hypoxia exposure was protective against development of the adhesion phenotype in NPFs.[21]

The ideal therapy for postoperative tissue fibrosis would be to prevent any tissue fibrosis from occurring, regardless of extent or severity, as a consequence of surgery. Historically, reduction of postoperative adhesions has been attempted by commercially available devices in various polymer formulations that interfere with tissue contact.[4] We believe, however, that complete and consistent prevention of postoperative tissue fibrosis, manifest as intra-abdominal adhesions, is more likely to be achieved by directing therapy at one or more of the steps involved in peritoneal healing. Dysregulation of the healing process, particularly at the early steps, can culminate in fibrotic, rather than normal resolution.

To date, no drug has been approved for the reduction or prevention of postoperative tissue fibrosis. Treatment options for reducing the extent and severity of adhesions presently include various medical devices approved worldwide. The established mechanism of action of these device treatments is to provide a physical barrier for separation of traumatized/injured tissues during the 3- to 5-day period required for remesothelialization of the peritoneal surface following surgery.[4]

L-alanyl-L-glutamine (Ala-Gln), a dipeptide comprised of alanine and glutamine joined by an amide bond, is presently being evaluated as a novel drug to prevent postoperative tissue fibrosis (adhesions). Administered by peritoneal instillation, Ala-Gln is taken up by transporters in the membrane of peritoneal cells. Glutamine is liberated from Ala-Gln by enzymatic cleavage upon entering the cell or in the extracellular space prior to uptake.[22] Glutamine becomes essential when catabolic conditions prevail, for example, following trauma associated with severe injury or surgical procedures. Large amounts of glutamine are released from muscle stores and are rapidly taken up by tissues, particularly by organs in the splanchnic region.[23-26] Plasma glutamine concentrations are thus observed to decrease significantly after trauma such as burns, major surgery, and in sepsis.[27]

Glutamine plays many roles in periods of stress, including use as a fuel source for rapidly dividing cells, a precursor for synthesis of nucleic acids, renal acid buffering, inducing cellular protection pathways, modulation of the inflammatory response, and prevention of organ injury.[28,29] Glutamine stimulates the formation of heat shock protein 70 in monocytes by enhancing the stability of messenger RNA (mRNA),[30-32] influences the redox potential of the cell by promoting the formation of glutathione,[33,34] and induces cellular anabolic effects by increasing the cell volume[35]; it regulates numerous effectors of cell growth and viability, including mitogen-activated protein kinase[36] and Jun (c-Jun N-terminal kinases), a stress-activated kinase,[37] inflammation-related agents such as NFkB, a cellular "first responder" to insult or injury,[38,39] interleukins and tumor necrosis factor-α [40], and mammalian target of rapamycin complex 1, which is a key regulator of cell growth and survival as a function of nutrient availability.[40] During wound healing, glutamine supports lymphocytes and monocytes, including tissue resident macrophages,[41,42] and helps drive oxidative phosphorylation at the level of the mitochondrion in epithelial cells.

Given that glutamine modulates numerous key effectors of cell growth and the inflammatory response to oxidative stress, we hypothesized that Ala-Gln might prevent postsurgical tissue fibrosis through regulation of early events in the response of peritoneal fibroblasts to surgical insults such as hypoxia and tissue trauma. In the present study, we assessed Ala-Gln's ability to inhibit the induction of two known mediators of the hypoxia-induced inflammatory response: HIF-1α and type 1 collagen, which are also established biomarkers of the adhesion phenotype in human peritoneal fibroblasts.[15,21] L-alanyl-L-glutamine effect on HIF-1α and type I collagen in NPFs was assessed under different conditions of hypoxic insult. The first condition featured Ala-Gln treatment at 3 dose levels under continuous hypoxia; the second involved exposure of cells to episodic hypoxia, followed by restoration of normoxia and immediate treatment with Ala-Gln.

Methods

Source and Culture of Human Peritoneal Fibroblasts

Normal parietal peritoneal tissues from the anterior abdominal wall lateral to the midline incision were excised from patients

undergoing laparotomy for pelvic pain. Samples were taken at the initiation of the surgery following entry into the abdominal cavity as previously described.[14,15] Normal peritoneal tissues were taken at a minimum of 7 cm from any observed tissue fibrosis. Participants did not have an active pelvic or abdominal infection and were not pregnant. All patients gave informed consent for tissue collection. All procedures were conducted under a protocol approved by the institutional review board of Wayne State University and done in full compliance with the Declaration of Helsinki and Principles of Good Clinical Practice.

Harvested tissue samples were immediately placed in standard media (Dulbecco modified Eagle medium [DMEM]; Invitrogen, Carlsbad, California) containing 10% fetal bovine serum (FBS), 2% penicillin, and streptomycin. Tissues were cut into small pieces in a sterile culture dish and transferred into another fresh T-25 flask with 3 mL of dispase solution (2.4 U/mL; GIBCO BRL, Life Technologies, Gaithersburg, Maryland). The flasks were incubated overnight at 37°C in an Environ-Shaker (LAB LINE Instruments, Melrose Park, Illinois). The samples were then centrifuged for 5 minutes at 1400*g*, transferred into a fresh T-25 flask with prewarmed DMEM medium, and placed in 37°C incubator (95% air and 5% CO_2); outgrowth of fibroblasts generally took 2 weeks. Once confluence was reached, the cells were transferred to 100-mm tissue culture dishes and cultured in standard media with 10% FBS. Thereafter, the confluent dishes were subcultured by trypsinization (1:3 split ratio). Studies were conducted using the same passage of cells to maintain comparability. Fibroblast cell lines have been extensively characterized in previous studies and have been shown to be pure and solely fibroblast cells.[43,44]

Treatment of Cells

In case 1 (continuous exposure to hypoxia), NPF cells (1.5×10^6) were cultured in 60 mm^2 tissue culture dishes under hypoxic (2% O_2) conditions for 0, 0.5, 1, 2, 4, 12, 24, or 48 hours in a 37°C incubator with or without Ala-Gln (1, 2, or 10 mM) and no restoration of normoxia. Cells were collected following their designated exposure time to hypoxia and analyzed for HIF-1α and collagen type 1a1 as described below (Protein Analysis).

In case 2 (episodic hypoxia), NPFs were exposed to hypoxia (2% O_2) for episodes of 0.5, 1, 2, 4, 12, 24, or 48 hours, then removed from hypoxia and placed under normoxic conditions (95% air and 5% CO_2), at which time they were immediately treated with or without Ala-Gln (1, 2, or 10 mM). Cells were then maintained in normoxia and collected at 6, 12, 24, 48, and 96 hours from the start of the experiment. Cell collection times all occurred after normoxia was restored; however, they were calculated from the start of the experiment, which was the start of hypoxia exposure.

In each case, exposure to hypoxia entailed placing cultures in an airtight modular incubator chamber (Billups-Rothenberg, Del Mar, California) via a positive infusion of 2% O_2 in a 5% CO_2-nitrogen gas balanced mixture. Hypoxic cultures were then placed in a standard humidified tissue incubator. Cell pellets were frozen at −80°C for later analysis. All experiments were performed in triplicate. Prior to the start of hypoxia, cells were sampled and analyzed for HIF-1α and collagen type 1a1 to establish prehypoxic levels of each adhesion biomarker.

Protein Analysis

Cell pellets were resuspended in phosphate-buffered saline and sonicated on ice for protein extraction. Total protein concentration of cell lysates was measured with the Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, Illinois) per the manufacturer's protocol. Equal concentration of protein was utilized in each enzyme-linked immunosorbent assay (ELISA). Protein was stored at −80°C and was thawed on ice before use in the ELISAs.

Enzyme-Linked Immunosorbent Assay

Levels of HIF-1α and type I collagen levels were determined utilizing the HIF-1α ELISA (Thermo Fisher Scientific, Waltham, Massachusetts) and the COL1A1 ELISA (LifeSpan Biosciences, Seattle, Washington), respectively, per the manufacturer's protocol. The detection limit of HIF-1α and COL1A1 ELISA is 50 and 31.25 pg/mL, respectively.

Statistical Analysis

Analyses were performed using SPSS v23.0 (IBM, Armonk, New York). Pair-wise differences in normally distributed variables in treatment and control groups were compared using 1-way analysis of variance with significant interactions further analyzed using Tukey post hoc analysis with Bonferroni correction.

Results

Prehypoxic Levels of Tissue Fibrosis Biomarkers in NPFs

Prehypoxic levels of each adhesion biomarker were determined to be 2.9 and 60.3 pg/mL per μg protein of HIF-1α and type 1 collagen, respectively (Table 1). These levels were determined by ELISA in cells prior to exposure to hypoxic conditions.

Effect of Continuous Hypoxia Exposure on Tissue Fibrosis Biomarkers in the Presence or Absence of Ala-Gln

Under continuous hypoxia (0.5-48 hours), a 300% to 400% increase in both tissue fibrosis biomarkers with increasing duration of hypoxia was observed in untreated controls (cells subjected to hypoxia but not treated with Ala-Gln; $P < .05$; Figure 1A and B, Tables 1 and 2). In fibroblasts treated with Ala-Gln, however, the levels of HIF-1α and type I collagen were substantially attenuated at all doses tested (1, 2, or 10 mM of Ala-Gln) compared to the corresponding controls not treated with Ala-Gln at each time point, $P < .007$ (Figure 1, Tables 1, 2, and 3.).

Table 1. Mean Levels of Type I Collagen and HIF-1α When Treated With Ala-Gln in the Presence of Continuous Hypoxia.

Ala-Gln Treatment	Hypoxia (Hours)	Type I Collagen (pg/μg Protein)	HIF-1α (pg/μg Protein)
No Ala-Gln	0	60.3 ± 3.5	2.9 ± 0.8
No Ala-Gln	0.5	195.6 ± 16.6	8.3 ± 0.7
No Ala-Gln	1	203.7 ± 15.9	10.3 ± 0.5
No Ala-Gln	2	222.0 ± 8.9	12.3 ± 0.2
No Ala-Gln	4	254.5 ± 8.0	11.1 ± 0.2
No Ala-Gln	12	274.3 ± 10.3	14.2 ± 0.4
No Ala-Gln	24	185.0 ± 8.6	11.2 ± 0.4
No Ala-Gln	48	172.7 ± 6.1	10.9 ± 0.3
1 mM Ala-Gln	0.5	168.7 ± 12.7	4.9 ± 0.7
1 mM Ala-Gln	1	152.2 ± 3.3	5.8 ± 0.5
1 mM Ala-Gln	2	154.0 ± 2.8	7.1 ± 0.2
1 mM Ala-Gln	4	163.2 ± 3.4	7.3 ± 0.5
1 mM Ala-Gln	12	177.0 ± 3.8	7.5 ± 0.5
1 mM Ala-Gln	24	173.4 ± 7.2	7.4 ± 0.2
1 mM Ala-Gln	48	160.9 ± 6.1	9.9 ± 0.2
2 mM Ala-Gln	0.5	112.7 ± 6.6	4.5 ± 0.6
2 mM Ala-Gln	1	123.7 ± 5.2	4.9 ± 0.5
2 mM Ala-Gln	2	131.6 ± 5.7	6.1 ± 0.3
2 mM Ala-Gln	4	95.0 ± 6.0	6.4 ± 0.5
2 mM Ala-Gln	12	96.6 ± 3 .9	6.6 ± 0.4
2 mM Ala-Gln	24	90.0 ± 1.6	6.6 ± 0.4
2 mM Ala-Gln	48	90.0 ± 2.7	9.7 ± 0.3
10 mM Ala-Gln	0.5	82.5 ± 5.4	3.8 ± 0.3
10 mM Ala-Gln	1	102.3 ± 10.8	3.5 ± 0.4
10 mM Ala-Gln	2	96.0 ± 5.1	5.0 ± 0.3
10 mM Ala-Gln	4	91.4 ± 7.1	5.2 ± 0.3
10 mM Ala-Gln	12	84.9 ± 4.3	5.6 ± 0.3
10 mM Ala-Gln	24	83.0 ± 5.7	5.1 ± 0.2
10 mM Ala-Gln	48	74.7 ± 2.9	9.5 ± 0.2

Abbreviation: Ala-Gln, L-alanyl-L-glutamine; HIF, hypoxia-inducible factor.

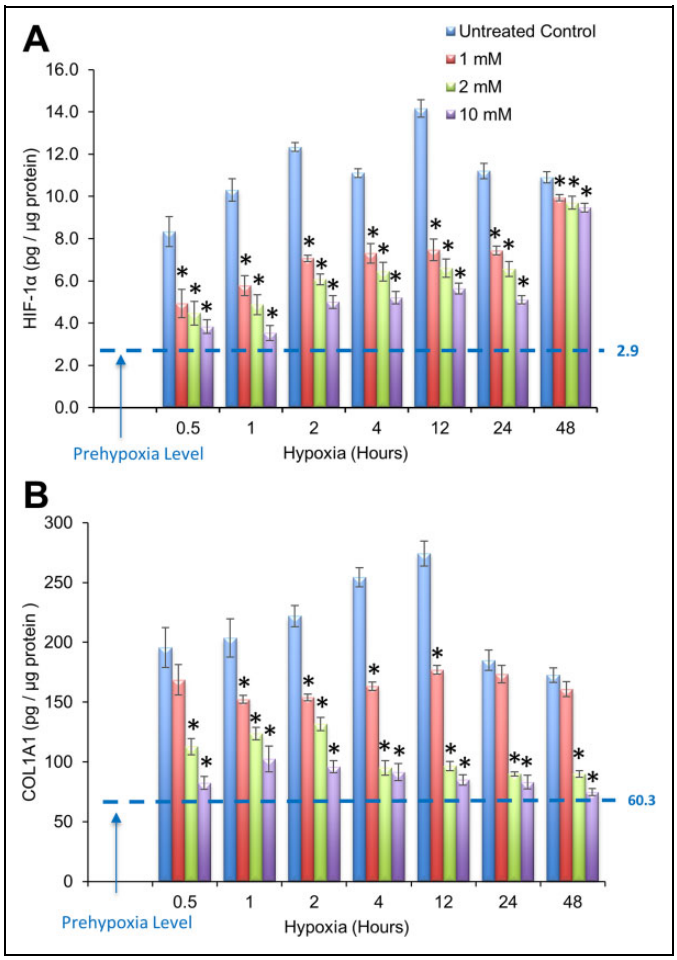

Figure 1. The effect of continuous hypoxia on postoperative adhesion marker levels is mitigated by L-alanyl-L-glutamine (Ala-Gln). To determine the effect of Ala-Gln on the levels of hypoxia-inducible factor (HIF-1α) and type I collagen, normal peritoneal fibroblasts were exposed to increasing durations of hypoxia (continuous exposure; 0.5-48 hours) in the presence of increasing doses of Ala-Gln (1, 2, or 10 mM). The levels of (A) HIF-1α and (B) type I collagen were assessed by enzyme-linked immunosorbent assay (ELISA). *$P < .05$ as compared to corresponding untreated control time point.

Effect of Episodic Hypoxia on Tissue Fibrosis Biomarkers in the Presence or Absence of Ala-Gln

Under conditions of episodic hypoxia (0.5-48 hours), fibroblasts not treated with increasing doses of Ala-Gln (1, 2, or 10 mM) upon restoration of normoxia exhibited substantially elevated levels of both HIF-1α and type I collagen compared to prehypoxic levels (Figures 2 and 3, Tables 1 and 2). Levels of both tissue fibrosis biomarkers declined over time upon restoration of normoxia and in the absence of Ala-Gln but remained elevated compared to prehypoxic levels out to 48 hours (Figures 2 and 3). In fibroblasts treated with the pharmacological dose of Ala-Gln (10 mM), a statistically significant reduction of each tissue fibrosis biomarker was observed up to 48 hours sampling time, regardless of hypoxic episode (Figures 2 and 3 and Tables 2 and 4). With lower doses of Ala-Gln (1 and 2 mM), a statistically significant reduction in HIF-1α was observed up to 24 hours sampling, but a statistically significant reduction in type 1 collagen up to 24 hours sampling was not observed consistently at the lowest dose of Ala-Gln ($P < .005$; Figures 2 and 3).

Effect of Episodic Hypoxia on HIF-1α in Fibroblasts Not Treated With Ala-Gln (0 mM)

Hypoxia-inducible factor 1α levels in untreated controls reached a maximum level of about 250% to 300% of prehypoxic controls at the 6-hour collection time point for all episodes of hypoxia. Peak HIF-1α levels gradually decreased in untreated controls (0 mM Ala-Gln) to levels approximately equivalent to those seen in fibroblasts not exposed to hypoxia when the episode of hypoxia was 1 hour or less. In cells exposed to 2 or 4 hours of hypoxia, however, HIF-1α levels remained substantially higher (about 140%) than levels seen in fibroblasts not exposed to hypoxia (Figure 2). The decline in HIF-1α levels over time in controls not treated with Ala-Gln may have been due to the short half-life of HIF-1α (Figure 2, Tables 2 and 4).

Table 2. Comparison of HIF-1α and Type I Collagen Levels After Treatment With Continuous Hypoxia (0.5-48 Hours) in the Presence of 1, 2, or 10 mM Ala-Gln-Treated NPFs.

	HIF-1α Levels—Continuous Hypoxia (*P* Values)			Type I Collagen Levels—Continuous Hypoxia (*P* Values)		
Hypoxia Treatment (hours) Plus Ala-Gln	Control vs 1 mM Ala-Gln	Control vs 2 mM Ala-Gln	Control vs 10 mM Ala-Gln	Control vs 1 mM Ala-Gln	Control vs 2 mM Ala-Gln	Control vs 10 mM Ala-Gln
0.5	.004	.002	.001	.089	.001	.0001
1	.0001	.0001	.0001	.005	.001	.001
2	.0001	.0001	.0001	.0001	.0001	.0001
4	.0001	.0001	.0001	.0001	.0001	.0001
12	.0001	.0001	.0001	.0001	.0001	.0001
24	.0001	.0001	.0001	.147	.0001	.0001
48	.005	.007	.002	.078	.0001	.0001

Abbreviations: Ala-Gln, L-alanyl-L-glutamine; HIF, hypoxia-inducible factor; NPF, normal peritoneal fibroblasts.

Table 3. Comparison of HIF-1α as well as Type I Collagen Levels in Untreated Control as Compared to 1, 2, or 10 mM Ala-Gln-Treated NPFs.

		HIF-1α—Episodic Hypoxia			Type I Collagen—Episodic Hypoxia		
		P Values			*P* Values		
Hypoxia (hours)	Collection Time Postinitiation of Hypoxia (hours)	Control vs 1 mM Ala-Gln	Control vs 2 mM Ala-Gln	Control vs 10 mM Ala-Gln	Control vs 1 mM Ala-Gln	Control vs 2 mM Ala-Gln	Control vs 10 mM Ala-Gln
0.5	6	.0001	.003	.002	.011	.0001	.0001
0.5	12	.0001	.0001	.0001	.403	.0001	.0001
0.5	24	.0001	.023	.0001	.0001	.0001	.0001
0.5	48	.025	.638	.105	.001	.0001	.0001
1	6	.012	.004	.016	.002	.0001	.0001
1	12	.001	.0001	.0001	.604	.065	.002
1	24	.002	.0001	.0001	.002	.001	.0001
1	48	.001	.047	.0001	.001	.292	.0001
2	6	.002	.002	.0001	.0001	.012	.0001
2	12	.0001	.0001	.0001	.041	.0001	.0001
2	24	.001	.0001	.0001	.0001	.0001	.0001
2	48	.0001	.013	.002	.004	.0001	.023
4	6	.003	.002	.0001	.019	.001	.008
4	12	.654	.0001	.0001	.001	.007	.009
4	24	.247	.086	.833	.001	.01	.04
4	48	.255	.432	.032	.636	.107	.007

[a]Cells were exposed to episodic hypoxia for 0.5, 1, 2, or 4 hours and moved to normoxia and were immediately treated with Ala-Gln.
Abbreviations: Ala-Gln, L-alanyl-L-glutamine; HIF, hypoxia-inducible factor; NPF, normal peritoneal fibroblasts.

Effect of Episodic Hypoxia on Type I Collagen in Fibroblasts Not Treated With Ala-Gln (0 mM)

Type I collagen levels in cells not treated with Ala-Gln were consistently at about 350% to 400% of prehypoxia levels by the 6-hour collection time point. These levels remained elevated to around 200% up to 48 hours observation, in untreated controls exposed to 2 hours of hypoxia or less (Figure 3, Tables 2 and 4).

Effect of Increasing Doses of Ala-Gln (1, 2, or 10 mM) on HIF-1α Under Episodic Hypoxia

A statistically significant suppression of HIF-1α levels in response to episodic hypoxia in the Ala-Gln-treated fibroblasts was observed for up to 24 hours sampling time at all doses of Ala-Gln as compared to untreated controls, in fibroblasts exposed to 0.5, 1, and 2 hours of hypoxia. In cells exposed to 4 hours of hypoxia, statistically significant attenuation of HIF-1α levels persisted only up to 12 hours and only in the 2 higher doses (Figure 2, Tables 2 and 4). At pharmacological levels (10 mM dose), the reduction in hypoxia-induced HIF-1α was statistically significant for up to 48 hours following hypoxia exposure times of 1 hour or longer. In cells exposed to 0.5 hours hypoxia, the reduction in HIF-1α levels achieved with the 2 lower doses at 48 hours observation was substantially lower than that in untreated controls and was roughly equivalent to prehypoxia levels, but was not statistically significant (Tables 2 and 4).

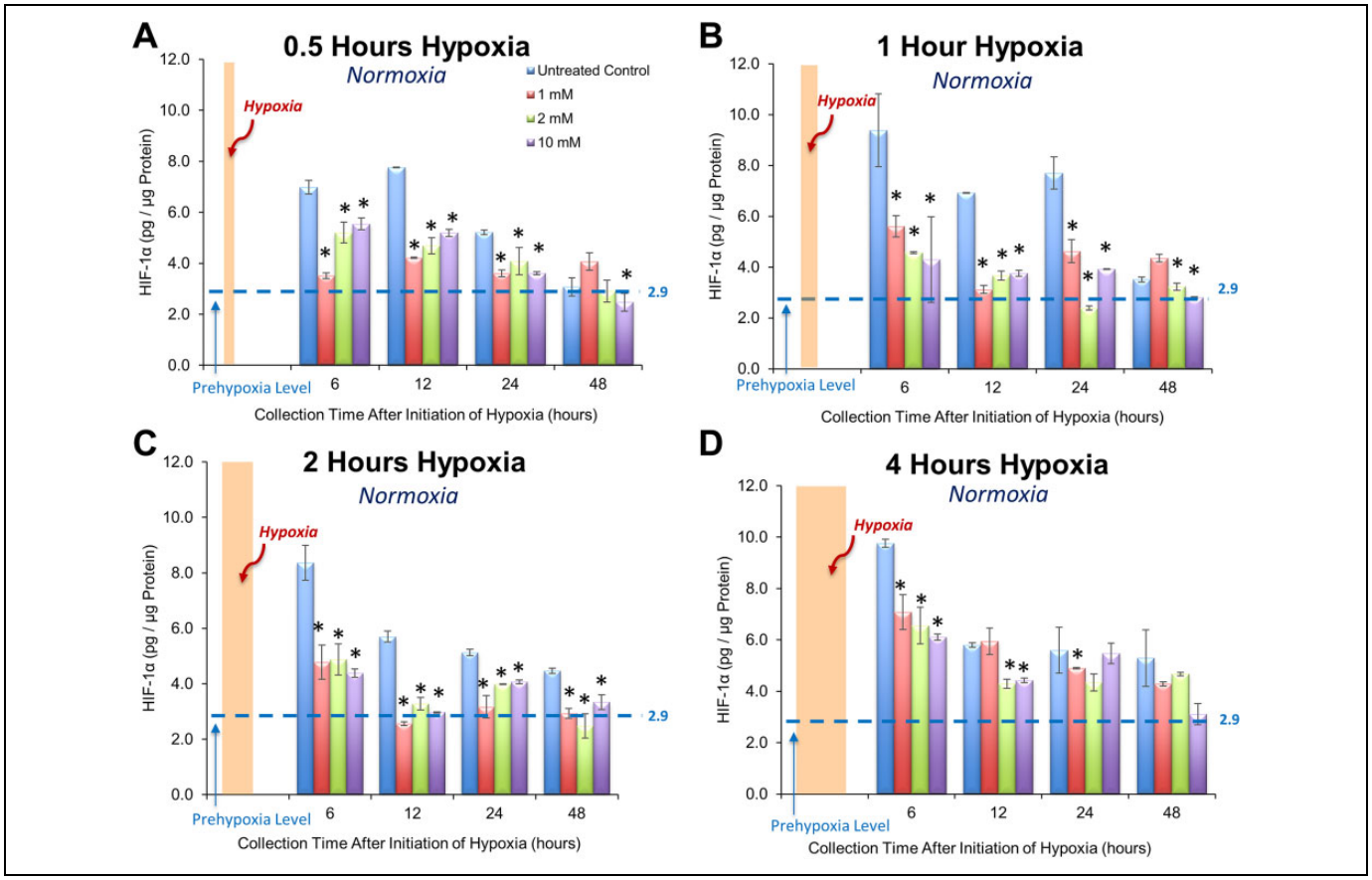

Figure 2. The early effects of Ala-Gln on HIF-1α levels after exposure to hypoxia. To assess the early effects of Ala-Gln after exposure to episodic hypoxia, cultures were treated under normal (95% air and 5% CO_2) or hypoxic (2% O_2) conditions for 0, 0.5, 1, 2, or 4 hours in a 37°C incubator. Following treatment with hypoxia at these time points, cells were placed under normoxic conditions and immediately treated with Ala-Gln (1, 2, or 10 mM). Cells were then collected at the 6, 12, 24, 48, and 96-hour time points from the initiation of hypoxia. *$P < .05$ as compared to corresponding untreated control time point.

Effect of Increasing Doses of Ala-Gln (1, 2, or 10 mM) on type I Collagen Under Episodic Hypoxia

For all episodes of hypoxia exposure, a statistically significant reduction in type 1 collagen was achieved by the 24-hour sampling time for all doses as compared to untreated controls (Figure 3, Tables 2 and 4). This effect persisted out to 48 hours sampling, except in cells exposed to the 4-hour episode of hypoxia, where statistically significant suppression of type I collagen was only achieved with the 10 mM dose (Figure 3, Tables 2 and 4).

Discussion

In the present study, cellular response to conditions of continuous and episodic intervals of hypoxia was assessed. Normal human peritoneal fibroblasts were an obvious choice as a test system for this study since they would be highly impacted by surgery involving the peritoneum and acutely involved in the process of peritoneal tissue fibrosis. The results indicate that Ala-Gln regulates cellular levels of 2 different mediators of the cellular response to insult or injury in peritonel cells that play an important role in development of postsurgical tissue fibrosis. A statistically significant reduction was observed in the levels of each of the biomarkers evaluated under episodic hypoxic exposure in Ala-Gln-treated fibroblasts compared to controls at all dose levels, throughout a 24-hour period of assessment for all episodes of hypoxia of 2 hours or less (Figure 1). These results are consistent with prior studies, in which we showed that hypoxia alone causes an elevation in protein levels of both HIF-1α and type I collagen in untreated controls (Figure 1).[8,9,21]

The persistent elevation of HIF-1α and type I collagen protein levels in untreated controls, even after restoration of normoxia, observed in the present study is consistent with prior observations of persistence in elevated mRNA levels of tissue fibrosis biomarkers as a consequence of hypoxia.[21,45,46] The present observations with respect to type I collagen seem to support an evolving hypothesis of hypoxia-induced alterations that cannot be reversed simply by restoring normoxia.[14,21]

Our results also establish that Ala-Gln's impact on the response of NPFs to hypoxia is observed to various extents at all doses of the drug, even when it was dosed after the

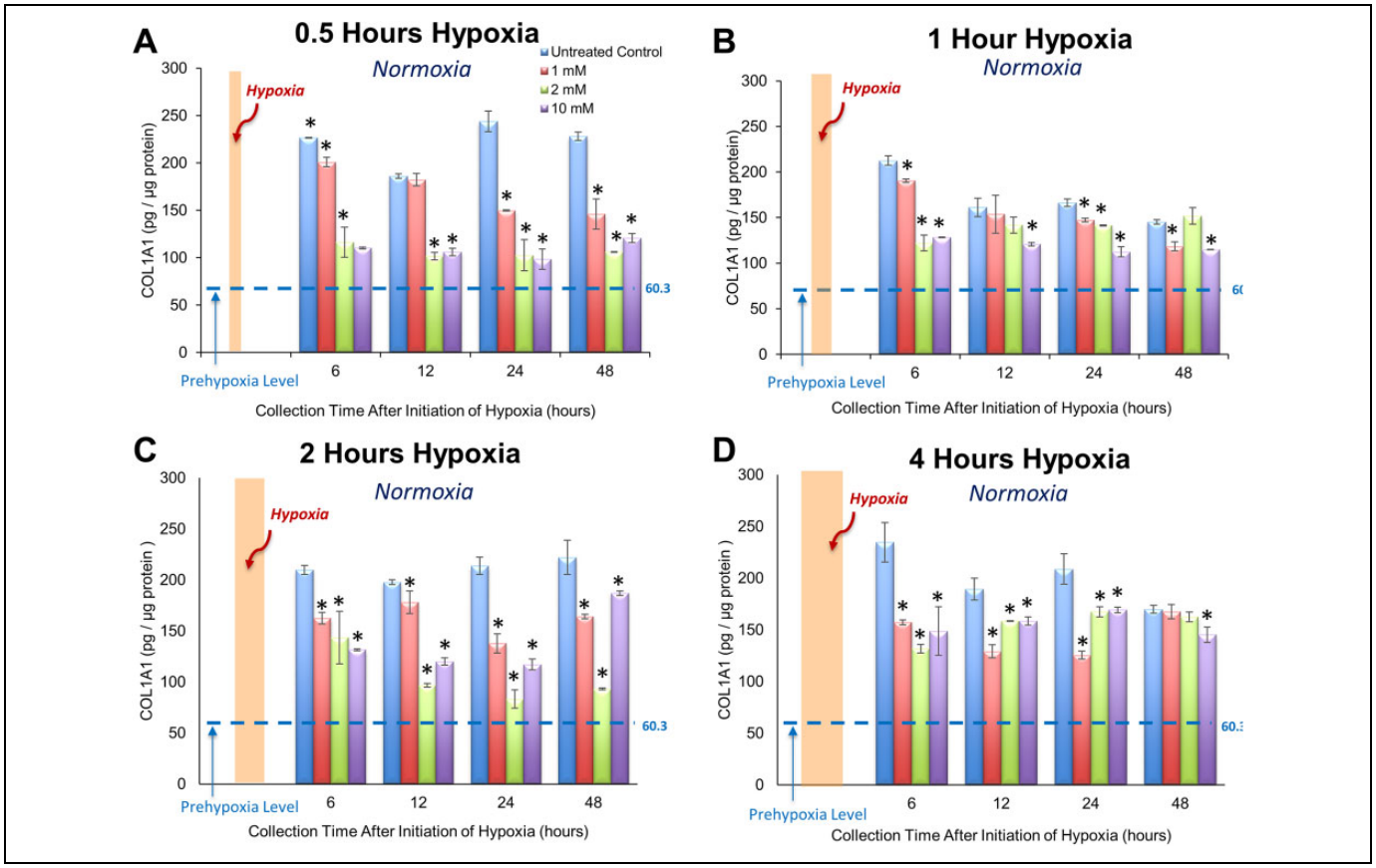

Figure 3. The early effects of Ala-Gln on type I collagen levels after exposure to hypoxia. To assess the early effects of Ala-Gln after exposure to episodic hypoxia, cultures were treated under normal (95% air and 5% CO_2) or hypoxic (2% O_2) conditions for 0, 0.5, 1, 2, or 4 hours in a 37°C incubator. Following treatment with hypoxia at these time points, cells were placed under normoxic conditions and immediately treated with Ala-Gln (1, 2, or 10 mM). Cells were then collected at the 6, 12, 24, 48, and 96-hour time points from the initiation of hypoxia. *$P < .05$ as compared to corresponding untreated control time point.

injurious stimulus (ie, hypoxia) was terminated. Such would be the case in surgical procedures and many acute injuries (Figure 2). Even a very brief period of hypoxia (eg, 30 minutes), prior to restoration of normoxia, resulted in elevation of HIF-1α and type I collagen protein levels when assessed at 6 hours following the start of the experiment (Figures 2 and 3). Not surprisingly, the highest dose (10 mM) achieved the most consistent and lasting suppression of both HIF-1α and type I collagen. This dose of Ala-Gln corresponds to about 6.7 mM Gln, which is approximately 10 times higher than normal circulating levels of glutamine.

The normal concentration of glutamine in plasma ranges from 0.5 to 0.7 mM. In catabolic states, or following trauma, glutamine is released from muscle stores and circulating levels can double.[47,48] The range of Ala-Gln concentrations (1, 2, 10 mM) used in this study corresponded roughly to normal physiologic levels, those observed following trauma, and pharmacological levels, respectively. Our results suggest that pharmacological levels of glutamine, as Ala-Gln, may be necessary to prevent postoperative tissue fibrosis in vivo. Pharmacological doses of Ala-Gln may be required not only to suppress initiation of the inflammatory cascade in response to hypoxia but also to maintain that effect for a sufficient period following insult to prevent fibrosis in the form of tissue fibrosis.

These results appear to correlate well with results of others who have demonstrated reduction of postsurgical tissue fibrosis by both glutamine and a specific inhibitor of HIF-1α in animal models. Glutamine's ability to mitigate the development of fibrosis due to injurious stimuli in vivo was demonstrated by San-Miguel et al in a rat colitis model.[49] These investigators reported that administration of glutamine (25 mg/kg infused for 7 days) to 2,4,6-trinitrobenzene sulfonic acid-treated rats resulted in early prevention of fibrosis development, an effect that correlated with inhibition of the colonic expression of collagens, TGFβ and other growth factors, and metalloproteinase inhibitor in colonic cells isolated from the test animals. Glutamine at pharmacological doses has been observed to prevent postoperative tissue fibrosis (adhesions) in humans as well as animals following surgeries involving the peritoneum.

Strowitzki and coworkers recently reported that a single intraoperative lavage with a HIF-1α inhibitor (20 mg/kg) reduced tissue fibrosis formation in a mouse model.[50] Their results in vivo were confirmed at the cellular level, where they observed that hypoxic upregulation of several biomarkers of

Table 4. Mean Type I Collagen and HIF-1α Levels in NPFs.[a]

Ala-Gln Treatment	Hypoxia (hours)	Collection Time Postinitiation of Hypoxia (hours)	Type I Collagen (pg/μg Protein)	HIF-1α (pg/μg Protein)
Baseline	0	0	60.3 ± 3.5	2.9 ± 0.8
No Ala-Gln	0.5	6	226.7 ± 0.4	7.0 ± 0.3
No Ala-Gln	0.5	12	186.1 ± 2.5	7.8 ± 0.02
No Ala-Gln	0.5	24	243.9 ± 10.8	5.2 ± 0.1
No Ala-Gln	0.5	48	228.1 ± 4.3	3.1 ± 0.4
No Ala-Gln	1	6	212.5 ± 5.2	9.4 ± 1.4
No Ala-Gln	1	12	161.2 ± 10.0	6.9 ± 0.02
No Ala-Gln	1	24	166.4 ± 4.2	7.7 ± 0.6
No Ala-Gln	1	48	145.3 ± 2.3	3.5 ± 0.1
No Ala-Gln	2	6	209.9 ± 4.5	8.4 ± 0.6
No Ala-Gln	2	12	197.7 ± 2.5	5.7 ± 0.2
No Ala-Gln	2	24	213.8 ± 8.3	5.1 ± 0.1
No Ala-Gln	2	48	222.1 ± 16.8	4.5 ± 0.1
No Ala-Gln	4	6	234.6 ± 19.1	9.8 ± 0.2
No Ala-Gln	4	12	189.4 ± 1 0.6	5.8 ± 0.1
No Ala-Gln	4	24	208.7 ± 14.8	5.6 ± 0.9
No Ala-Gln	4	48	169.8 ± 3.8	5.3 ± 1.1
1 mM Ala-Gln	0.5	6	201.0 ± 4.8	3.5 ± 0.1
1 mM Ala-Gln	0.5	12	182.3 ± 6.6	4.2 ± 0.03
1 mM Ala-Gln	0.5	24	149.8 ± 0.7	3.6 ± 0.1
1 mM Ala-Gln	0.5	48	146.1 ± 16.0	4.1 ± 0.3
1 mM Ala-Gln	1	6	190.4 ± 2.0	5.6 ± 0.4
1 mM Ala-Gln	1	12	153.7 ± 20.7	3.1 ± 0.2
1 mM Ala-Gln	1	24	147.4 ± 2.2	4.6 ± 0.5
1 mM Ala-Gln	1	48	118.4 ± 5.0	4.3 ±0.1
1 mM Ala-Gln	2	6	162.5 ± 5.6	4.8 ± 0.6
1 mM Ala-Gln	2	12	178.1 ± 11.1	2.6 ± 0.1
1 mM Ala-Gln	2	24	137.8 ± 9.5	3.2 ± 0.4
1 mM Ala-Gln	2	48	164.2 ± 2.2	2.9 ± 0.2
1 mM Ala-Gln	4	6	157.2 ± 2.3	7.1 ± 0.7
1 mM Ala-Gln	4	12	129.1 ± 6.4	5.9 ±0.5
1 mM Ala-Gln	4	24	125.5 ± 3.8	4.9 ± 0.03
1 mM Ala-Gln	4	48	167.5 ± 6.8	4.3 ± 0.1
2 mM Ala-Gln	0.5	6	116.3 ± 16.0	5.2 ± 0.4
2 mM Ala-Gln	0.5	12	101.6 ± 4.0	4.7 ± 0.3
2 mM Ala-Gln	0.5	24	102.6 ± 16.6	4.1 ± 0.5
2 mM Ala-Gln	0.5	48	105.9 ± 0.5	2.9 ± 0.4
2 mM Ala-Gln	1	6	122.2 ± 8.7	4.6 ± 0.04
2 mM Ala-Gln	1	12	141.8 ± 8.7	3.7 ± 0.2
2 mM Ala-Gln	1	24	141.4 ± 0.7	2.4 ± 0.1
2 mM Ala-Gln	1	48	151.8 ± 9.0	3.2 ± 0.1
2 mM Ala-Gln	2	6	143.5 ± 25.9	4.9 ± 0.6
2 mM Ala-Gln	2	12	96.6 ± 1.8	3.3 ± 0.2
2 mM Ala-Gln	2	24	83.2 ± 9.0	4.0 ± 0.02
2 mM Ala-Gln	2	48	93.1 ± 1.0	2.5 ± 0.4
2 mM Ala-Gln	4	6	131.8 ± 4.3	6.6 ± 0.7
2 mM Ala-Gln	4	12	158.5 ± 0.4	4.3 ± 0.2
2 mM Ala-Gln	4	24	167.5 ± 4.9	4.3 ± 0.3
2 mM Ala-Gln	4	48	162.6 ± 4.7	4.7 ± 0.1
10 mM Ala-Gln	0.5	6	110.2 ± 1.0	5.5 ± 0.2
10 mM Ala-Gln	0.5	12	106.1 ± 4.0	5.2 ± 0.1
10 mM Ala-Gln	0.5	24	98.5 ± 10.7	3.6 ± 0.1
10 mM Ala-Gln	0.5	48	120.6 ± 4.7	2.5 ± 0.3
10 mM Ala-Gln	1	6	128.2 ± 0.5	4.3 ± 1.7
10 mM Ala-Gln	1	12	120.8 ± 1.9	3.8 ± 0.1
10 mM Ala-Gln	1	24	112.4 ± 5.7	3.9 ± 0.02
10 mM Ala-Gln	1	48	114.9 ± 0.2	2.8 ± 0.04
10 mM Ala-Gln	2	6	131.6 ± 1.0	4.4 ± 0.2
10 mM Ala-Gln	2	12	120.0 ± 3.5	3.0 ± 0.02
10 mM Ala-Gln	2	24	117.1 ± 5.3	4.1 ± 0.1
10 mM Ala-Gln	2	48	187.1 ± 2.2	3.3 ± 0.3
10 mM Ala-Gln	4	6	148.7 ± 23.5	6.1 ± 0.1
10 mM Ala-Gln	4	12	158.4 ± 3.9	4.4 ± 0.1
10 mM Ala-Gln	4	24	169.2 ± 2.8	5.5 ± 0.4
10 mM Ala-Gln	4	48	145.2 ± 7.4	3.1 ± 0.4

[a]Cells were exposed to episodic hypoxia for 0.5, 1, 2, or 4 hours and moved to normoxia and were immediately treated with Ala-Gln.
Abbreviations: Ala-Gln, L-alanyl-L-glutamine; HIF, hypoxia-inducible factor; NPF, normal peritoneal fibroblasts.

inflammation, including HIF-1α and TGF-β, was blunted in normal murine peritoneal fibroblasts in vitro upon treatment with micromolar doses of a HIF-1α inhibitor.

We report herein that glutamine suppresses the hypoxic upregulation of HIF-1α and type I collagen in normal human peritoneal fibroblasts exposed to continuous or episodic hypoxia in vitro. To our knowledge, these results are the first report of Ala-Gln suppression of HIF-1α levels, induced in response to hypoxia, in normal human peritoneal fibroblasts.

The inflammatory response to injury that culminates in postsurgical tissue fibrosis is a complex cascade of events orchestrated, at least in part, by HIF1α. Our results suggest that Ala-Gln may have therapeutic value in preventing postsurgical tissue fibrosis by inhibiting HIF-1α induction, a critical early event in peritoneal fibroblast response to injurious stimuli (such as hypoxia) that can be sustained during surgical procedures. This mechanism of action for glutamine is novel and provides support for an equally innovative approach to preventing postoperative tissue fibrosis. Further studies, both in vitro and in vivo, are required to completely elucidate the relationship between Ala-Gln's suppression of postinsult levels of tissue fibrosis biomarkers in NPFs and prevention of postsurgical tissue fibrosis in humans and animals.

Conclusion

Work was performed at Wayne State University School of Medicine, Detroit, Michigan

Declaration of Conflicting Interests

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