Glucose-dependent insulino-tropic polypeptide (GIP) directly affects collagen fibril diameter and collagen cross-linking in osteoblast cultures

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Glucose-dependent insulino-tropic polypeptide (GIP) is absolutely crucial in order to obtain optimal bone strength and collagen quality. However, as the GIPR is expressed in several tissues other than bone, it is difficult to ascertain whether the observed modifications of collagen maturity, reported in animal studies, were due to direct effects on osteoblasts or indirect through regulation of signals originating from other tissues. The aims of the present study were to investigate whether GIP can directly affect collagen biosynthesis and processing in osteoblast cultures and to decipher which molecular pathways were necessary for such effects. MC3T3-E1 cells were cultured in the presence of GIP ranging between 10 and 100 pM. Collagen fibril diameter was investigated by electron microscopy whilst collagen maturity was determined by Fourier transform infrared spectroscopy (FTIRM). GIP treatment resulted in dose-dependent increases in lysyl oxidase activity and collagen maturity. Furthermore, GIP treatment shifted the collagen fiber diameter towards lower value but did not significantly affect collagen heterogeneity. GIP acted directly on osteoblasts by activating the adenylyl cyclase–cAMP pathway. This study provides evidences that GIP acts directly on osteoblasts and is capable of improving collagen maturity and fibril diameter.

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Introduction

Glucose-dependent insulino-tropic polypeptide (GIP) is an important gastro-intestinal hormone synthesized and secreted into the blood stream by intestinal endocrine K cells after ingestion of a mixed meal [1–3]. In humans, fasting plasma GIP is ranged between 12 and 92 pM and increases to 35–235 pM postprandially [4]. To induce a biological response, GIP binds to a specific glucose-dependent insulino-tropic polypeptide receptor (GIPR) expressed in several tissues including bone tissue [5–9]. However, despite expression of its receptor at the surface of osteoblasts, little is known about the osseous effects of the GIP/GIPR pathway. Previously, we reported that the deletion of the Gipr gene in mouse resulted in dramatic alterations of trabecular bone microarchitecture [10]. We also evidenced that bone strength and material properties were markedly reduced in this animal model with a dramatic 16% decrease in collagen maturity [11]. Furthermore, treatment of healthy Copenhagen rats with a stable GIP agonist resulted in a 13% increase in collagen maturity [12]. Taken together, these results suggest that the GIP/GIPR pathway is involved in the control of collagen quality in the bone matrix. However, as the GIPR is expressed in several tissues other than bone, it is difficult to ascertain whether the observed modifications in collagen quality are due to direct effects on osteoblasts or indirect through regulation of signals originating from other tissues. Bone tissue is considered to be a two-component material made primarily of mineral (poorly crystalline hydroxyapatite) and organic (collagen type I) matrices [13]. The mineral component confers stiffness to the bone tissue [14,15] whilst collagen confers a degree of tensile strength, ductility and toughness [16,17]. Collagen type I biosynthesis by osteoblasts is a complex process and includes intracellular post-translational modifications, assembly of procollagen chains and secretion [18]. In the extracellular space, C-terminal and N-terminal propeptides are cleaved resulting in collagen fibril formation. The ultimate processing of collagen fibrils is represented by enzymatic cross-linking. Cross-linking stabilizes the collagen network and is due to oxidative deamination of ε-amino groups of specific lysine and hydroxylsine residues by lysyl oxidase, forming aldehyde moieties [19]. These aldehydes are highly reactive and lead to formation of covalent crosslinks that are critically required for the formation of functional mature collagen. The assembly of collagen molecules into fibrils has been described as entropy driven, similar to that occurring in other self-assembling proteins such as microtubules or actin filaments [20]. However, data obtained from genetically-modified animals revealed that several other molecules, present in the extracellular bone matrix,
may regulate collagen deposition and fibril formation. Among them, glycosaminoglycan-containing molecules such as biglycan and decorin are suspected to play a major role in collagen deposition and fibril formation as demonstrated by abnormal collagen fibrils in deficient animals [21,22]. Furthermore, evidences have been provided that lysyl oxidase activity is also a pre-requisite for normal collagen deposition and fibril organization [23].

The aims of the present study were to investigate whether GIP directly affects collagen deposition and maturation in osteoblast cultures and to decipher what molecular pathways were necessary for such effects.

**Material and methods**

**Reagents**

Alpha-Minimum Essential Medium Eagle (α-MEM), fetal bovine serum (FBS), bovine calf serum, penicillin, and streptomycin were purchased from Lonza (Levallois-Perret, France). α-Aminopropionitrile (BAPN) was purchased from VWR (Fontenay-sous-bois, France). All other chemicals were purchased from Sigma-Aldrich (Lyon, France) when otherwise stated.

**Peptide synthesis**

D-alâ-glucose-dependent insulinotropic polypeptide (GIP) was synthesized in our peptide supplier Genecust (Dudelange, Luxembourg). Briefly, GIP was sequentially synthesized using standard solid-phase Fmoc protocols [24] using Wang resin. Substitution of alanine at the N-terminal position 2 with its (D) isomer was performed to ensure better resistance to serum dipeptidylpeptidase-4 as described elsewhere [25]. The peptide was then purified by reverse-phase HPLC (purity >95%) using a SinoChrom ODS-BP column (4.6 × 250 mm) and subsequently characterized by electrospray ionization mass spectroscopy (ESI-MS) using a LCMS-2010 device (Shimadzu Corporation, Duisburg, Germany). Mass spectra were collected using full ion scan mode over (ESI-MS) using a LCMS-2010 device (Shimadzu Corporation, Duisburg, Germany). Mass spectra were collected using full ion scan mode over

**Cell culture**

MC3T3-E1 cells were phenotypically normal osteoblasts derived from fetal mouse calvaria and differentiate in culture resulting ultimately in bone-like extracellular matrix [27]. MC3T3-E1 cells were purchased from American type culture collection (ATCC, Teddington, UK). Cells were grown and expanded at a ratio 1:4 in propagation medium containing αMEM supplemented with 5% FBS, 5% bovine calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere enriched with 5% CO2 at 37 °C. Then cells were starved in αMEM supplemented with 0.5% BSA for 16 h and incubated for 15 min in αMEM supplemented with 0.5% bovine serum albumin and 1 mM IBMX prior to stimulation with GIP. After 45 min, cells were washed with ice-cold PBS, incubated in lysis buffer and centrifuged at 12,000 g for 10 min. Supernatants were collected and stored at −80 °C until use. Cyclic AMP determination was performed with a fluorometric commercially available kit (R&D Systems Europe, Abingdon, UK) according to the manufacturer’s recommendations.

**Lysyl oxidase (LOX) activity**

LOX activity was assessed in the cell culture supernatant and in the cell layer after 13 days of culture, as this period of time corresponds to the highest expression of lysyl oxidase [23] using a fluorometric assay developed by Palamakumbura et al. [28]. At day 12, the differentiation medium was replaced by phenol red-free DMEM containing 0.5% bovine serum albumin, 50 μg/mL ascorbic acid, 2.5 mM β-glycerophosphate and various concentrations of GIP. After 24 h, cell culture supernatants were collected, centrifuged at 3000 rpm for 30 min at 4 °C, aliquoted and stored at −80 °C until use. The cell layer was extracted in urea buffer as reported in [29]. Then, supernatants were incubated at 37 °C for 30 min in the presence of 1.2 M urea, 50 mM sodium borate (pH 8.2), 1.3 nmol H2O2, 1 UI/mL horseradish peroxidase, 10 mM dianisidopentane. 10 μM Amplifluor™ red and ± 0.5 mM BAPN in opaque 96 well plates. At the end of the incubation period, the plate was placed on ice and fluorescence was read using a M2 microplate reader (Molecular devices, St. Grefoire, France) with excitation and emission wavelength set up at 563 nm and 587 nm, respectively. As BAPN is a specific lysyl oxidase inhibitor, the residual amine oxidase activity evidenced in the presence of BAPN was subtracted from the activity observed in the absence of BAPN. Lysyl oxidase activity was reported as the fold change versus CTRL of the pooled supernatant and cell layer fractions.

**Western blotting**

MC3T3-E1 cells were washed in cold PBS and lysates were made by using a lysis buffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 3 mM Na3VO4, protease inhibitor cocktail and 1% Nonidet P-40. Samples were spun at 13,000 rpm for 30 min at 4 °C, the supernatant was collected and protein concentration was determined by BCA assay (ThermoScientific, Bребières, France). Samples (20 μg per lane) were run on a 10% acrylamide gel and blotted onto a PVDF membrane. The membranes were washed in Tris buffered saline (TBS) and blocked with 5% bovine serum albumin. Samples were incubated overnight with the anti-GIPR (reference sc-98795, Santa Cruz Biotechnology, Heidelberg, Germany) or β-actin (Sigma-Aldrich). Subsequently membranes were washed in TBS and incubated with the appropriate secondary antibodies coupled to HRP (R&D Systems Europe). Immunoreactive bands were visualized using an ECL kit (Amersham, UK).

**Fourier transformed infrared microscopy (FTIRM)**

For collagen cross-link analysis, the same osteoblast cultures used for LOX activity were processed. Osteoblast cultures were fixed in absolute ethanol, scraped off the culture dish and transferred onto BaF2 windows where they were air-dried. The integrity of the collagen extracellular matrix was verified by comparing the obtained FTIRM spectrum with those of commercial collagen. Spectral analysis was performed using a Bruker Vertex 70 spectrometer (Bruker optics, Ettlingen, Germany) interfaced
with a Bruker Hyperion 3000 infrared microscope equipped with a standard single element Mercury Cadmium Telluride (MCT) detector. Infra-red spectra were recorded at a resolution of 4 cm⁻¹, with an average of 32 scans in transmission mode. Background spectral images were collected under identical conditions from the same BaF₂ windows at the beginning and end of each experiment to ensure instrument stability. For FTIRM analysis, 20 spectra were acquired for each condition and analyzed with the Opus Software (release 6.0, Bruker). Water vapor was corrected prior to baseline correction. Spectra were then subjected to second derivative spectroscopy and curve fitting routines using a commercial available software package (Grams/Al 8.0, Thermofisher scientific, Villebon sur Yvette, France) as described previously [30]. The collagen maturity index was determined as the relative ratio of pyridinium trivalent (Pyr, mature collagen) to dehydrodihydroxylysinonorleucine divalent (deH-DHLNL, new collagen) collagen cross-links using their relative (Pyr, mature collagen) to dehydrodihydroxylysinonorleucine divalent (deH-DHLNL, new collagen) cross-links in the extracellular matrices (Fig. 2). The area of the 1376 cm⁻¹ peak, corresponding to the Amide I band (1585–1725 cm⁻¹). The sulfated proteoglycan-to-matrix ratio was calculated as the ratio of the 1660 cm⁻¹ peak, corresponding to SO₃ symmetric stretching vibration of sulfated proteoglycan [34] to the Amide I band.

Transmission electron microscopy (TEM)

After 13 days of culture, osteoblasts and their produced extracellular matrix were rinsed with 0.1 M cacodylate buffer (pH 7.4) and fixed in 2.5% glutaraldehyde in cacodylate buffer. Then cultures were post-fixed with 1% osmium tetroxide/1% potassium ferrocyanide and dehydrated in a graded series of ethanol prior to embedding in epoxy resin. Ultrathin sections were cut with a diamond knife, contrasted with 3% uranyl acetate and observed with a Jeol JEM 1400 (Jeol, Croisy sur Seine, France) operating with an accelerating voltage of 120 KeV. As represented Fig. 1A, GIPR is expressed in MC3T3-E1 osteoblast cells. GIP dose dependently induced a significant cAMP stimulation to reach 4 pmoles/well at concentrations of 1 nM and above and an EC₅₀ of 96pM (Fig. 1B). We next investigated LOX activity in the presence of GIP ranged between 10 and 100 µM (Fig. 1C). LOX activity was augmented in cultures treated with as low as 10 pM GIP (1.1-fold increase, p < 0.05) as compared with untreated cells. Furthermore, LOX activity was significantly increased by 2.2-fold (p < 0.001), 3.6-fold (p < 0.001) and 3.9-fold (p < 0.001) in the presence of 20 pM, 50 pM and 100 pM GIP, respectively.

We then examined the extent of mature and immature collagen cross-links in the extracellular matrices (Fig. 2). The area of the 1690 cm⁻¹ subband, representative of the deH-DHLNL immature collagen cross-link, was dose-dependently augmented in GIP-treated culture (Fig. 2A). A similar pattern was observed with the area of the 1660 cm⁻¹, a peak representative of pyridinoline collagen cross-links. The augmentation of the 1660 cm⁻¹ area was even more marked was computed and only fibrils with an AR < 1.2 were considered with low probability of local tilting. The minor diameter of fitted ellipse was selected as the value of fibril diameter as described by Starborg et al. [35]. More than 800 fibrils were measured per condition. The diameter distribution was then analyzed with a lab-made routine using ImageJ 1.45s and Excel 2010. Rapidly, the frequency of occurrence of an i diameter was calculated as follows:

\[
F_i = 100 \times \frac{N_i}{N_t}
\]

where \(N_i\) represents the number of fibrils with the diameter \(i\) and \(N_t\) represents the total number of fibrils. The frequency distribution, as a function of fibril diameter, was plotted and the following parameters were deduced from this distribution: \(D_{\text{peak}}\), corresponding to the most frequently observed fibril diameter, \(D_{\text{mean}}\) the average fibril diameter and \(D_{\text{width}}\), the width of the histogram at half maximum of the peak.

Statistical analysis

Each experiment has been replicated at least 3 times. Results were expressed as mean ± standard error of the mean (SEM). Non-parametric Mann–Whitney U-test was used to compare the differences between the groups using the Systat statistical software release 13.0 (Systat software Inc., San Jose, CA). Differences at \(p < 0.05\) were considered to be significant.

Results

GIP affects lysyl oxidase (LOX) activity and collagen maturity

As represented Fig. 1A, GIPR is expressed in MC3T3-E1 osteoblast cells. GIP dose dependently induced a significant cAMP stimulation to reach 4 pmoles/well at concentrations of 1 nM and above and an EC₅₀ of 96pM (Fig. 1B). We next investigated LOX activity in the presence of GIP ranged between 10 and 100 µM (Fig. 1C). LOX activity was augmented in cultures treated with as low as 10 pM GIP (1.1-fold increase, \(p < 0.05\)) as compared with untreated cells. Furthermore, LOX activity was significantly increased by 2.2-fold (\(p < 0.001\)), 3.6-fold (\(p < 0.001\)) and 3.9-fold (\(p < 0.001\)) in the presence of 20 pM, 50 pM and 100 pM GIP, respectively.

We then examined the extent of mature and immature collagen cross-links in the extracellular matrices (Fig. 2). The area of the 1690 cm⁻¹ subband, representative of the deH-DHLNL immature collagen cross-link, was dose-dependently augmented in GIP-treated culture (Fig. 2A). A similar pattern was observed with the area of the 1660 cm⁻¹, a peak representative of pyridinoline collagen cross-links. The augmentation of the 1660 cm⁻¹ area was even more marked

![Fig. 1. GIP affects LOX activity and collagen maturity. (A) GIPR expression has been assessed by western blotting in three independent MC3T3 cell culture. (B) cAMP production in response to increasing doses of GIP. (C) LOX activity is dose-dependently increased in the presence of GIP. *: \(p < 0.05\) and **: \(p < 0.001\) vs. untreated cultures.](image-url)
at higher dose of GIP (Fig. 2B). The collagen maturity index was not significantly different between vehicle- and 10 pM GIP-treated cultures ($p = 0.075$) (Fig. 2C). But, at higher concentrations of GIP, significant augmentations in this index were observed with 73% ($p = 0.047$), 127% ($p = 0.009$) and 131% ($p = 0.009$) increases in this parameter with 20 pM, 50 pM and 100 pM, respectively. We also assessed whether GIP treatment affected the proteoglycan content of the extracellular matrix. The PG-to-matrix and sulfated PG-to-matrix ratios were unaffected by the presence of GIP (Figs. 2D and E).

**GIP affects collagen fibril diameters**

As represented in Fig. 3A, it seemed that fibril diameters were reduced in the presence of 100 pM GIP. The frequency distribution of fibril diameters also appeared shifted towards lower diameter in the presence of 100 pM GIP (Fig. 3B). Indeed, $D_{\text{mean}}$, the mean diameter, and $D_{\text{peak}}$, the most often observed diameter, were dramatically reduced, in a dose-dependent manner, in the presence of GIP with lower diameters observed at the highest tested concentrations (Table 1). On the other hand, $D_{\text{width}}$ that characterizes heterogeneity in fibril diameters was not significantly different between untreated and GIP-treated cultures at all tested concentrations (Table 1).

**Adenylyl cyclase activity is required for GIP-mediated increases in LOX activity and collagen crosslinking**

As binding of GIP to its receptor in osteoblasts leads to activation of adenylyl cyclase and production of cAMP, we ascertained whether the observed increases in LOX activity and collagen cross-linking required a functional adenylyl cyclase. The use of the adenylyl cyclase inhibitor

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**Fig. 2.** GIP affects collagen maturity. (A) Amount of deH-DHLNL, (B) amount of Pyr and (C) collagen maturity index, expressed as the ratio of Pyr/deH-DHLNL (1660 cm$^{-1}$/1690 cm$^{-1}$), are dose-dependently increased in the presence of GIP. (D) Total proteoglycan content and (E) sulfated proteoglycan content in the extracellular bone matrix. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ vs. untreated cultures.

**Fig. 3.** GIP affects collagen fibril diameter. (A) TEM photographs of untreated (left) and 100 pM GIP-treated (right) cells. Bar = 200 nm. Inset represents a higher magnification of collagen fibrils. Bar in inset = 100 nm. (B) Distribution frequency of fibril diameter between untreated (black) and 100 pM GIP-treated (gray) cells. Note the shift toward lower values for fibril diameter in GIP-treated cells.
2′,5′-dideoxyadenosine (DDA) significantly decreased the production of cAMP in GIP-treated cultures (Fig. 4A). Furthermore, DDA had no influence on LOX activity in vehicle-treated cultures (p = 0.507), Fig. 4B). On the other hand, this activity was reduced by 25% in the presence of DDA. As a consequence, addition of DDA in 100 pM GIP-treated cultures resulted in significant increases in Dmean and Dwidth but not Dpeak. The addition of DDA in GIP-treated cultures led to values for Dpeak and Dwidth not significantly different from those observed in control cultures.

**Discussion**

Previously, in animal models of GIPR deficiency or stable GIP analog treatment, evidences have been provided that maturity of the collagen bone matrix was modified by the GIP/GIPR pathway [11,12]. However, due to the pleiotropic expression of GIPR, it was unclear whether this modification of collagen maturity resulted from direct action of the GIP/GIPR pathway on bone cells. In the present study, using a validated model of osteoblast cultures, we evidenced a significant stimulation of cAMP production in response to increasing doses of GIP, significant augmentations in LOX activity and collagen maturity in GIP-treated cultures and significant reductions in collagen fiber diameters. These modifications of LOX activity and collagen properties were partly due to stimulation of adenylyl cyclase and production of cAMP as evidenced by alterations of these modifications in the presence of the adenylyl cyclase inhibitor, 2′,5′-dideoxyadenosine.

Adenylyl cyclase activity is required for GIP-mediated modifications of the collagen matrix

As represented Fig. 5, the fibril diameter seemed unmodified in control cultures treated with or without DDA. On the other hand, the fibril diameter in GIP-treated cultures seemed higher in the presence of DDA. The frequency distribution of fibril diameter was unaffected in control cultures by the presence of DDA whilst the frequency distribution in GIP-treated cultures in the presence of DDA was broadened and shifted toward higher values. Not surprisingly, GIP treatment led to a decrease in Dpeak and Dmean as reported above whilst Dwidth was unmodified (Table 2). In control cultures, the addition of DDA did not affect any of the distribution frequency parameters. However, addition of DDA in GIP-treated cultures resulted to significant increases in Dmean and Dwidth but not Dpeak. The addition of DDA in GIP-treated cultures led to values for Dpeak and Dwidth not significantly different from those observed in control cultures.

### Table 1
Properties of collagen fibrils in the presence of GIP.

<table>
<thead>
<tr>
<th>GIP (pM)</th>
<th>Dpeak (nm)</th>
<th>Dmean (nm)</th>
<th>Dwidth (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>30.3 ± 0.7*</td>
<td>29.7 ± 0.5**</td>
<td>12.3 ± 2.6</td>
</tr>
<tr>
<td>20</td>
<td>28.5 ± 0.3**</td>
<td>29.3 ± 0.8**</td>
<td>10.4 ± 1.2</td>
</tr>
<tr>
<td>50</td>
<td>27.9 ± 0.8**</td>
<td>27.9 ± 0.2**</td>
<td>9.6 ± 0.5</td>
</tr>
<tr>
<td>100</td>
<td>27.8 ± 0.7**</td>
<td>27.8 ± 0.5**</td>
<td>9.5 ± 0.7</td>
</tr>
</tbody>
</table>

*: p < 0.05 and **: p < 0.01 vs. cells cultured in the absence of GIP.

**Table 2**

Properties of collagen fibrils in the presence of an adenylyl cyclase inhibitor. Osteoblast cultures were treated with vehicle or 100 pM GIP in the presence of 50 μM DDA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dpeak (nm)</th>
<th>Dmean (nm)</th>
<th>Dwidth (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>32.0 ± 0.7</td>
<td>32.7 ± 0.6</td>
<td>11.8 ± 1.3</td>
</tr>
<tr>
<td>Vehicle + DDA</td>
<td>32.0 ± 1.2</td>
<td>32.7 ± 1.1</td>
<td>12.5 ± 0.8</td>
</tr>
<tr>
<td>GIP</td>
<td>28.0 ± 0.2*</td>
<td>27.7 ± 0.2*</td>
<td>10.4 ± 0.8</td>
</tr>
<tr>
<td>GIP + DDA</td>
<td>29.1 ± 0.8*</td>
<td>30.2 ± 0.2**</td>
<td>13.8 ± 0.6*</td>
</tr>
</tbody>
</table>

*: p < 0.05 and **: p < 0.01 vs. cells cultured in the absence of GIP.

* p < 0.05 vs. vehicle.

Fig. 4. GIP effects on LOX activity and collagen maturity are due to a cAMP-dependent mechanism. (A) cAMP stimulation in 100 pM GIP culture is abolished by the use of 50 μM 2′,5′-dideoxyadenosine (DDA). (B) Increase in LOX activity observed in the presence of 100 pM GIP is significantly reduced in DDA-treated cultures. White bars = untreated or GIP-treated cultures in the absence of DDA, black bars = untreated or GIP-treated cultures in the presence of DDA. (C) Amount of deH-DHLNL, (D) amount of Pyr and (E) collagen maturity index were significantly reduced in 100 pM GIP-treated cultures in the presence of DDA. White bars = untreated or GIP-treated cultures in the absence of DDA, black bars = untreated or GIP-treated cultures in the presence of DDA. *: p < 0.05 vs. cells cultured in the absence of DDA, **: p < 0.01 and ***: p < 0.001 vs. cells cultured in the absence of DDA.
Collagen must be cross-linked to exhibit the normal physical properties of tensile strength. The final enzymatic step of collagen cross-linking is ensured by LOX, an enzyme with amine oxidase activity, generating \( \delta \)-aminoadipic-\( \delta \)-semialdehyde and \( \delta \)-aminoadipic-\( \delta \)-semialdehyde from hydroxylysine and lysine residues, respectively [39, 44]. These aldehydes then spontaneously react with other aldehydes or unmodified lysine and hydroxylysine residues to form a variety of intra- and intermolecular cross-links in collagens. LOX is synthesized in osteoblasts as a pro-enzyme and excreted in the extracellular environment as a pro-enzyme. The pro-enzyme is then processed by procollagen C-proteinases in the extracellular environment to give rise to the active 32 kDa enzyme and an 18 kDa propeptide [37, 38]. Additionally, four LOX-like enzymes (LOXL1-4) have been identified and present significant sequence identity with mature LOX [39-44]. In MC3T3-E1 cells, LOX, LOXL1, LOXL3 and LOXL4 have been shown to be the prominent form at the RNA level [45]. Furthermore, LOXL1, LOXL3 and LOXL4 also present amine oxidase activities [46-48]. In the present study, the LOX activity was determined in conditioned media of MC3T3-E1 cells stimulated with various concentrations of GIP. We observed a significant increase in this parameter; however it is unclear whether this observed increase is related to increases in only LOX or LOXL activities or a combination of both. However, it is worth noting that in lox \(-/-\) animals, significant reductions in LOXLs at the RNA levels were observed [49]. Nevertheless, the increase in LOX activity after GIP stimulation was accompanied by an augmentation in collagen cross-linking as determined spectroscopically. The ratio between the subbands located at 1660 cm\(^{-1}\) and 1690 cm\(^{-1}\) is a cheap and validated methodology for the determination of collagen cross-linking [32]. This methodology required second-derivative spectral deconvolution and curve fitting in order to resolve underlying bands of the amide I peak. However, it can be applied either on tissue sections or cell culture material as we performed in the present study [50-56].

Not only collagen maturation but also collagen fibril diameter was affected by GIP treatment. Indeed, we observed a significant 17% reduction in the mean fibril diameter in the presence of 100 pM GIP. However, the same heterogeneity in fibril diameter distribution was observed between control and GIP-treated cultures as determined by the \( D_{90\%} \) parameter. Collagen fibril assembly was thought to be an entropy-driven process as observed for microtubules and actin filament. On the other hand, some non-collagenous proteins have been implicated in fibrils assembly. Among them, biglycan and decorin, two proteoglycans of the extracellular matrix have been suggested. Biglycan and decorin are composed of a protein core with leucin-repeat repeats attached to chains of chondroitin sulfate. To determine the content of total proteoglycans in the extracellular matrix we chose a spectroscopic approach and we used the 1376 cm\(^{-1}\) band instead of the conventional 940–1140 cm\(^{-1}\) region. Indeed, although the 940–1140 cm\(^{-1}\) area comprises the C–OH and C–C ring vibrations of the carbohydrate in proteoglycans, this region might also contain information from plastic particles detached during extracellular matrix scraping and ethanol residues (mainly between 940 and 1050 cm\(^{-1}\)). Recently, Rieppo et al. demonstrated that the ratio of the 1376 cm\(^{-1}\) band over the entire integrated area of the Amide I peak represented a good indicator of total proteoglycan content [33]. Furthermore, these authors made also the demonstration of the usefulness of the 1064 cm\(^{-1}\) band as a marker of sulfated proteoglycans [33]. The overall proteoglycan content and the sulfated proteoglycan content of the extracellular matrix were determined and appeared unaffected by GIP treatment. As such, it is unlikely, although we did not determine the expression of biglycan and decorin in the extracellular matrix, that the observed reductions in collagen fibril diameters were due to a modified pattern of proteoglycan expression.

The GIP receptor has been shown to be expressed and functional in several osteoblastic cell lines (Saos-2, TE-85, MG-63) and primary murine osteoblasts [6,8]. The GIP receptor is also expressed on MC3T3-E1 cells and addition of GIP in the cultures resulted in a significant stimulation of cAMP with an \( EC_{50} \) around 100 pM. The observed \( EC_{50} \) is

![Fig. 5. GIP effects on collagen fibril diameter are due to a cAMP-dependent mechanism.](image-url)
relatively close to the physiological circulating plasma levels of GIP encountered after a meal. The use of a specific adenyl cyclase inhibitor, 2′,5′-dideoxyadenosine was capable of reducing by 88%, the production of cAMP in MC3T3-E1 cells in response to GIP. Moreover, DDA did not only abolish the cAMP response but also contributed to significant reductions in LOX activity (−25%) and collagen cross-linking (−36%). Furthermore, collagen deposition was also affected by the use of DDA in GIP-treated cultures and significant augmentations in the mean ﬁbril diameter and distribution heterogeneity were evidenced. Taken together, these data support a direct role for a GIPR-adenyl cyclase-cAMP axis in the control of extracellular matrix properties in response to GIP. Noteworthy is the residual cAMP activity in the presence of GIP/ DDA. Indeed, in this condition, MC3T3-E1 exhibited still a 7% increase in cAMP as compared with untreated cells. One could wonder why we did not use higher doses of DDA to completely abolish the rise in cAMP. As a preliminary experiment, we used several doses of DDA and 50 μM was the highest dose usable as higher concentration led to cell death in the culture. Of course, we cannot rule out that other intracellular pathways, independent of adenyl cyclase activation, might also be stimulated by the binding of GIP to the GIPR and as such might contribute to the observed modiﬁcations of extracellular matrix.

In conclusion, the present study supports a direct role for the GIPR-adenyl cyclase-cAMP in the control of extracellular matrix deposition and properties in osteoblasts. GIP-treated osteoblasts presented with augmented LOX activity and collagen cross-linking as well as modiﬁcation of collagen deposition.

References


